(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 154 454**
A1

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: 85301173.2

(22) Date of filing: 21.02.85

(51) Int. Cl.⁴: C 12 N 15/00
C 12 P 19/34, C 12 P 21/00
C 07 K 15/04, A 61 K 39/015
C 07 K 17/00
//C12R1:90

(30) Priority: 22.02.84 GB 8404692
26.09.84 GB 8424340

(43) Date of publication of application:
11.09.85 Bulletin 85/37

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

(71) Applicant: THE WELLCOME FOUNDATION LIMITED
183-193 Euston Road
London NW1 2BP(GB)

(72) Inventor: Holder, Anthony Arthur
Langley Court
Beckenham Kent BR3 3BS(GB)

(72) Inventor: Sandhu, Jasbir Singh
Langley Court
Beckenham Kent BR3 3BS(GB)

(72) Inventor: Odink, Karel Gerrit
Eggstrasse 19
CH-4402 Frenkendorf(CH)

(72) Inventor: Lockyer, Michael James
Langley Court
Beckenham Kent BR3 3BS(GB)

(72) Inventor: Riveros-Moreno, Valentina
Langley Court
Beckenham Kent BR3 3BS(GB)

(74) Representative: Garrett, Michael et al,
The Wellcome Foundation Limited Group Patents and Agreements Langley Court
Beckenham Kent BR3 3BS(GB)

(54) **Cloning of DNA for protozoal antigens.**

(57) The present invention relates to the cloning of the P.195 gene of *Plasmodium falciparum*, its use in expressing the P.195 protein and the use of P.195 in vaccines for the treatment or prophylaxis of malaria.

EP 0 154 454 A1

0154454

Cloning of DNA for Protozoal Antigens

The present invention relates to a cloned sequence of DNA, and fragments thereof, of Plasmodium falciparum which may be used to provide antigenic peptides for use in malaria vaccines.

Malaria is an increasing health problem throughout the third world. Several hundred million people suffer from the disease and the most acute form, caused by the protozoan parasite Plasmodium falciparum, kills over a million children each year in Africa alone. An effective immunity against the asexual multiplication of the parasite in the blood-stream would prevent the clinical disease. Prevention of the re-invasion of red blood cells by an effective immune response against the invasive form, the merozoite, should interrupt this cycle.

In a rodent malarial model it has been shown that a protein antigen synthesised within the mature intraerythrocytic form, the schizont, and expressed on the surface of the merozoite, can be purified and that vaccination with this antigen can generate protective immunity against Plasmodium yoelii (Holder, A.A. and Freeman, R.R. Nature 294, 361-364 (1981)). This antigen has an apparent molecular weight (MW) of 230,000 and is proteolytically processed in vivo such that discrete fragments of the antigen are present on the merozoite surface (Holder and Freeman, 1981 supra; Holder, A.A. and Freeman, R.R. Parasitology 88, 211-219 (1984a)).

By "molecular weight" is meant the apparent relative molecular weight as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and standard molecular weight markers. The molecular weight of the antigenic proteins of the invention may thus be conveniently determined by the techniques described by U.K. Laemmli, Nature, (1970) 227, 680-685. Convenient standard molecular weight markers include, for example, spectrin heterodimer (2.2×10^5 M.W.), β-galactosidase (1.16×10^5 MW), phosphorylase b (9.3×10^4 MW), bovine serum albumin (6.8×10^4 MW), aldolase (3.9×10^4 MW), triose phosphate isomerase (2.7×10^4 MW) and lysozyme (1.5×10^4 MW).

When the protein was used to vaccinate mice the protection was adjuvant dependent and it appeared to be provided by a cell mediated effector pathway (Freeman, R.R. and Holder, A.A. Clin. Exp. Immunol. 54, 609-616 (1983a)),

although a monoclonal antibody against the protein has been shown to confer passive protection upon mice (Majariam W.R. et al., J. Immunol. 132, 3131-3137 (1984)).

Analogous protein antigens have also been described in other Plasmodium species. A polyvalent antiserum raised against the purified 230,000 MW antigen of P. yoelii cross-reacted by immunofluorescence with the blood stage forms of all other species of Plasmodium tested (Holder and Freeman, 1984a, supra). In Plasmodium chabaudi the antigen was identified by Western blotting as a 250,000 MW species which may also be processed (Holder, A.A. et al., Mol. Biochem. Parasitol. 9, 191-196 (1983)). Monoclonal antibodies specific for the 250,000 MW P. chabaudi protein were shown to confer passive protection against P. chabaudi challenge in mice (Boyle, D.B. et al., Infect Immun. 38, 94-102 (1982)). A monoclonal antibody against a 230,000 MW protein of Plasmodium knowlesi agglutinated merozoites and thereby inhibited parasite invasion of red cells in vitro (Epstein N. et al., J. Immunol. 127, 212-217 (1981)), and this protein of P. knowlesi is processed in vivo to a series of fragments expressed on the merozoite surface (David P.H. et al., Mol. Biochem. Parasitol. 11, 267-282 (1984)).

In P. falciparum, polyvalent antiserum against the P. yoelii 230,000 MW protein antigen cross-reacted with a 195,000 MW antigen (hereinafter referred to as the P.195 protein) (Holder et al., (1983) supra). The biosynthesis of this antigen, which appears to be a glycoprotein (Howard, R.J. et al., Mol. Biochem. Parasitol. 11, 349-362 (1984)) takes place within the schizont form of the parasite and at the end of the intra-erythrocytic stage the antigen is proteolytically processed into discrete fragments (Holder, A.A. and Freeman, R.R. J. Exp. Med. 156, 1528-1538, (1982); Hall, R. et al., Mol. Biochem, Parasitol. 11, 61-80 (1984a)). On the surface of the merozoites (released into the serum at the end of the intra-erythrocytic stage), the protein is fully processed, with three discrete fragments of P. 195 having molecular weights of about 83,000, 42,000 and 19,000 being present. These three fragments are the major surface antigens of merozoites and are strongly recognised by human immune serum (Freeman, R.R. and Holder, A.A. J. Exp. Med. 158, 1647-1653 (1983b), Holder, A.A. and Freeman, R.R. J. Exp. Med. 160, 624-629 (1984b)).

The term 'P.195' is used herein to denote a protein of between 1.8 and 2.3 x 10^5 MW which is localised in the erythrocytic schizont form of a Plasmodium falciparum parasite and which is processed in vivo into discrete fragments of

approximately 8.3 x 10^4, 4.2 x 10^4 and 1.9 x 10^4 MW, associated with the surface membranes of the merozoite form of the parasite.

Within P. falciparum P.195 may exhibit some structural polymorphism as detected by the degree of binding of specific monoclonal antibodies or by small differences in its apparent molecular weight (McBride, J.S. et al., Science 217, 254-257 (1982); McBride, J.S. et al., Trans. Roy. Soc. Trop. Med. Hyg. 78, 32-34 (1984); Hall, R. et al., (1984a) supra). However it will be appreciated by those skilled in the art that these antigens are homologous and that differences demonstrated in particular antigenic determinants may not be important in the broader sense of an immune response in an animal. It has been shown that Saimiri monkeys immunised with P.195 were protected against challenge infection (Perrin, L.H. et al., J. Exp. Med. 160, 441-451 (1984); Hall, R. et al., Nature 311, 379-382 (1984b)).

The term 'epitope' as used herein denotes an immunogenic determinant of an immunogenic molecule, the immunogenic determinant comprising a molecular configuration capable of eliciting a protective immune response in a susceptible animal, when presented in a suitable form.

It will also be appreciated that P.195 is subject to allelic variation, wherein different strains of P. falciparum express proteins different from, but substantially similar to any one P.195 protein from any one strain.

A comparison of the P.195 gene from the P. falciparum strain described herein with the corresponding gene from a strain isolated by Thaithong et al. (Thaithong, S., Beale, G.H., Fenton, B., McBride, J., Rosario, V., Walker, A. and Walliker, D., Trans. Roy. Soc. Trop. Med Hyg. (1984) 78, 242-245) by Southern blotting showed structural polymorphism detectable by nucleic acid hybridisation (R.T. Schwartz).

For the reasons outlined above, it is believed that the P. falciparum P.195, or an antigenic fragment thereof, is of value for use in a blood-stage malaria vaccine.

In order to obtain this protein or an antigenic fragment thereof,such as those produced in vivo and which are present on the merozoite surface, in large quantities and in a relatively pure form, it would be desirable to identify the sequence of the DNA in the gene coding for the expression of this protein in P.

falciparum. After identification of the sequence, it would be desirable to reproduce the immunologically effective parts of the protein molecule, either by cloning this sequence in a suitable vector and expression in a suitable host, or by chemical synthesis of the amino acid sequence corresponding to the identified sequence.

We have now discovered that it is possible to clone the DNA sequence that substantially encodes the above described antigen (P.195) from P. falciparum and that the functional antigen or fragments thereof can be obtained by incorporating said cloned DNA into a suitable vector which, in an appropriat host, is capable of expressing the antigen or peptides comprising at least one epitope thereof.

According to one feature of the present invention we therefore provide a cloned DNA sequence substantially encoding the P.195 protein of P. falciparum, or peptides comprising at least one epitope thereof.

The term 'cloned' is used herein to indicate any DNA sequence that has been substantially synthesised outside of its natural host.

The term 'peptide' as used herein pertains to any molecular structure composed essentially of amino acids comprising more than 2 amino acids. It will be appreciated that P.195 is a peptide by this definition.

It will be appreciated that the DNA sequences of this invention may correspond to naturally-occurring sequences, or they may be related to such sequences by mutation, including single or multiple base substitutions, deletions, insertions and inversions, always provided that the DNA molecule comprising such a sequence is capable of being expressed as a peptide carrying at least one epitope of the P.195 protein of P. falciparum.

The invention will be described in more detail hereinafter with reference to the accompanying drawings in which:

Figure 1 shows the base sequence of a stretch of P. falciparum DNA containing the gene encoding P.195 and the amino acid sequence for which it codes.

Figure 2 illustrates a cDNA restriction map of the P.195 gene.

Figure 3 illustrates a restriction map of the P.195 genomic sequence.

Figure 4 illustrates the construction of plasmids used in the course of exemplifying the invention.

Figures 1A to 1I show (in sequence) the nucleotide sequence of the P.195 gene, the amino acid sequence for which it codes and stretches of sequence at either end of the coding sequence. The lower line of each double line of letters represents nucleotides according to convention, while the upper line shows the amino acid sequence for which the open reading frame codes, the letters representing amino acids according to convention. The sequence was determined by methods described herein and it will be appreciated that, while it has been determined as accurately as possible within the margins of experimental error, there may be some variation in the P.195 genetic sequence.

Figure 2 shows the gene, including sequence in the cDNA clones extending beyond either end of the coding sequence, at the top of the figure (thick line) with important restriction enzyme sites marked. All restriction enzyme codes in this figure are according to convention. Other restriction enzyme sites are indicated on parallel lines below, to simplify the diagram. The blocked-in lines at the bottom of the figure indicate the positions relative to the P.195 gene from which various plasmid inserts derive, all being cDNA inserts except G1, which represents a genomic insert. X and Y indicate the putative 5' and 3' ends of the P.195 coding sequence, respectively.

Figure 3 illustrates a restriction map of the stretch of genomic DNA including the P.195 gene. The scale is in Kbp and takes as its reference point a Hind III restriction site of the P.195 gene indicated by the bold capital H in the figure. The other restriction enzyme sites are coded as follows; E(EcoR1), R(Rsa1), A(Alu1), M(Mbo1), Pv(PvuII), N(Nde1), T(Taq1), B(BamH1) and P(Pst1). The stretches of sequence shown below the restriction map are those that were found to hybridise to the specific clone indicated. The number in brackets is the cDNA clone to which the segment hybridised, the number always corresponding to the relevant pPFc clone. Where there is also a number outside the brackets followed by two of the above-indicated letters, this indicates that the probe used was less than the whole clone, the number indicating the length of the fragment in Kbp and the letters indicate the restriction enzymes used to generate the said fragment.

Figure 4 illustrates the construction of 2 plasmids used to express fragments of P.195 DNA. pWRL507 has a number of characteristic restriction sites as indicated in the figure, and characteristic gene functions wherein Ptrp, trpE are a promoter and anthranilate synthetase I respectively, Amp^R confers ampicillin resistance and Tet^R confers tetracycline resistance. pXY460 also possesses characteristic restriction sites as indicated, a promoter, (Ptac) a gene coding for β-galactosidase (lac^Z) and a gene conferring ampicillin resistance, Amp^R.

The above-mentioned DNA sequence may be characterised as having substantially all or part of the sequence shown in Figure 1, subject to the conditions noted above and further subject to considerations of experimental errors consequent upon determining such a sequence.

The above-mentioned DNA sequence may also be characterised as having substantially the restriction maps as shown in Figures 2 and 3, determined as described herein.

The genetic sequence was determined by the chemical cleavage method (Maxam, A., and Gilbert, W Meth. Enzymol 65, 499 (1980)) or by the dideoxy method (Sanger, et al., (1977) Proc. Natl. Acad. Sci., 74, 5463-5467) after sub-cloning fragments of the target DNA into the bacteriophage cloning vectors M13mp8 and M13mp9 (Messing, J and Vieira, J, (1982) Gene 19, 269-276).

Analysis of the sequence shown in Figure 1 reveals a likely start codon (AUG) at position 216 followed by an open reading frame of a further 1654 codons. The calculated molecular weight of the peptide gene product is 189, 953. The start codon is followed by a putative signal sequence of 18 codons coding for an amino acid sequence which would be cleaved off the protein before it matures. Nucleotides 447-527 code for alternate repeats of the tripeptide sequences serine-glycine-glycine and serine-valine-alanine occuring within the 83,000 MW fragment of P.195 (Example 6). The distribution of some of the amino acids within the translated sequence is asymmetric. For example, of the 19 cysteine residues, two are in the putative signal peptide and eleven are in the C-terminal 97 amino acids (the 42,000 MW Fragment). Eleven tripeptide sequences of the structure Asn-X-Ser or Thr (where X can be any of the common amino acids except proline) have been identified which are potential N-glycosylation sites.

Using the sequence data shown in Figure 1 it will be appreciated that a peptide corresponding to any part of the sequence may be synthesised using for example, the methods described by Merrifield, R.B., and Marglin, A. (Ann. Rev. Biochem., 39, 841 et seq. (1970)).

Thus, in a further embodiment of the invention, we provide a synthetic peptide comprising at least one epitope of P.195.

The term 'synthetic' as used herein relates to peptides produced by a chemical method as described above, for example.

The identification and cloning of a fragment of the DNA sequence coding for P.195 may be carried out, for example, as follows. Whole messenger RNA (mRNA)

was first extracted from synchronous cultures of P. falciparum by treating the cells with detergent, precipitating the mRNA by ethanol treatment and centrifugation and purifying by chromatography on oligo-dT cellulose. The substantially pure product was then used to synthesise copy DNA (cDNA) using reverse-transcriptase and DNA polymerase. After purification, the cDNA was inserted into a plasmid which was then introduced into a host by transformation. In order to ascertain which clones in the resulting 'library' contained relevant DNA inserts, a probe was isolated by centrifuging P. falciparum mRNA through a sucrose gradient and characterising the fractions by in vitro translation. A fraction found to encode P.195 was rendered radioactive, after limited alkaline hydrolysis, with polynucleotide kinase and ^{32}P-ATP. Using this probe in a colony hybridisation experiment, several clones were found to hybridise to it, some of them strongly. These were then sorted into families by cross-hybridisation and one of each family was made radioactive by nick-translation with DNA polymerase and α-^{32}P-dATP. Assuming these probes represent parts of the DNA sequence for P.195, they should hybridise to a mRNA of not less than 5,300 bases long in a total extract of P. falciparum mRNA, as this is the minimum estimated length of mRNA necessary to code for a protein of 195,000 MW.

Probes that recognised such mRNA (Northern blot procedure) were then characterised further by incorporation into a vector which, in a suitable host, was capable of expressing the cDNA sequence as a fusion peptide. To ensure expression of this cDNA, the fragments were treated with exonuclease prior to incorporation into the vector in order to give randomised reading frames. Peptides expressed were probed with polyvalent rabbit serum raised against P.195. The DNA fragment referred to above was detected using such a procedure.

With regard to the cloning of full length cDNA for P.195 there are now available several methods for cDNA synthesis to represent the entire mRNA (eg Heidecker, G. and Messing, J. (1983) Nucleic Acid Res. 11, 4891-4906). Cloning may also be carried out by the use of total genomic DNA digest 'libraries' which are prepared readily, and the relevant sequence, or fragments thereof, detected in such a library by the use of the above-describ d fragment as a 'probe' (for example, Odink, K.G. et al., Mol. Biochem. Parasitol. (1984) 10, 55-66). A proportion of sequences found by this technique may be full length cDNA sequences as required, though many will be fragments of such a sequence. In order to determine which clones in the library represent a part of the DNA

sequence desired, a method known as 'chromosome walking' (Hadfield, C., Focus 5, 1-5 (1983) Bethesda Res. Labs.) may be employed, which method entails the use of known fragments (probes) to detect other fragments by cross-hybridisation. These freshly located sequences may then, themselves, be used as probes and, in this way, the whole sequence of DNA substantially encoding P.195 may be identified and cloned. Using such procedures, a restriction map characteristic of the DNA sequence may also be prepared.

The construction of a physical map of the gene for P.195 in genomic DNA by restriction endonuclease cleavage, gel electrophoresis, transfer to nitrocellulose or polyamide membranes and hybridisation to specific probes derived from the cloned DNA is extremely useful to confirm and facilitate the orientation and position of cDNA and genomic clones. In addition a comparison of the restriction sites within the cDNA clones with those in the genome can be used to detect features of the gene which may not be present in the mRNA from which the cDNA is synthesised. An example would be the presence of discontinuities within the coding sequence, introns, which may be spliced out of the transcribed RNA by specific splicing events. One such possible intron of about 700 b.p. has been located by the above techniques between nucleotides 221 and 313 of the coding sequence (Fig.1), that is, between the MboI(M) and Hind III sites at these positions (Fig.3).

As indicated above, P.195 is processed in vivo into discrete fragments including the fragments referred to above. These fragments may prove to be of considerable value in providing immunity against malaria, and the DNA sequences for such fragments represent an important embodiment of the present invention, particularly since such sequences are generally likely to be better capable of expression in suitable vectors than the DNA sequence for the entire protein.

Thus, in a further aspect of the present invention we provide a cloned DNA sequence substantially encoding any one of the P.195 fragments occurring in vivo.

Those fragments of the naturally occurring P.195 most likely to elicit an immune response in a susceptible host are those that are present on the merozoite surface.

In a yet further aspect of the present invention there is provided a cloned DNA sequence substantially encoding any one of the P.195 fragments occurring on the surface membrane of a P. falciparum merozoite in vivo.

To locate the position of the processing fragments (Holder and Freeman, 1984b, supra) in the linear gene sequence, one direct approach is to purify the fragments and determine a partial amino acid sequence which can then be compared with the translated gene sequence. This has proved to be feasible for the 83,000 MW fragment which appears to be specifically shed from the merozoite, possibly during the process of red cell invasion, and accumulates in the supernatants of in vitro cultures. Sequencing of the 20 amino-terminal residues of the 83,000 MW fragment of P.195 has also shown that the corresponding coding sequence is located from nucleotides 273 to 332 in Figure 1, thus positioning this fragment within the gene.

The position of the 42,000 MW fragment was determined by the use of monoclonal antibodies in conventional manner and is described in Example 7. It has been established that the coding sequences for the 42,000 MW and 83,000 MW fragments are at opposite ends of the gene.

Experiments to demonstrate allelic variation in the P.195 gene have shown that the greatest conservation occurs in the region 5' to the Hind III site (in the 83,000 MW fragment) and in the 3' non-coding region (Figure 2). The most highly conserved sequence is at the 3' end of the gene corresponding to about 130 amino acid residues at the carboxy terminal of the 42,000 MW fragment, suggesting that this fragment may comprise at least one useful epitope.

Thus in a further embodiment of the invention we provide a DNA sequence corresponding to the 42,000 MW fragment of P.195.

The DNA sequence according to the invention may also be used to produce viruses containing the DNA sequence. For example, a strain of vaccinia virus (Tk^-) unable to confer upon infected cells the ability to grow on media not containing hypoxanthine is used to infect a tissue culture. The tissue culture may then be transformed with the P.195 gene, or fragment(s) thereof, linked to a Tk^+ genetic determinant. Some of the subsequent viral progeny will have such transforming sequences in the form of inserts in their genomes. These can then be selected by their ability to confer upon tissue culture cells the ability to grow on media devoid of hypoxanthine. Such colonies as do grow are then furth r selected for production of P.195, or peptides comprising at least one

epitope thereof, for example by use of a relevant monoclonal antibody such as F111.2. Such vaccinia strains can then be used to infect animals susceptible to malaria, as the new vaccinia strain will cause production of an immunogenic malarial peptide(s). Thus, in a yet further aspect of the invention we provide a non-pathogenic virus provided with the DNA sequence according to the invention which may be used to provide immunity to malaria in a susceptible vertebrate host.

It will be appreciated that such vaccines are also readily capable of providing immunity to other infections, such as smallpox, diphtheria, hepatitis B, rabies, herpes simplex virus, whooping cough and the like. Thus, the invention also provides a non-pathogenic virus as defined above, further capable of providing immunity to other infection(s) which may be administered jointly, or individually, together with any other vaccine.

Based on the above-described characterisation of the DNA sequence for P.195, the cloning of any desired fragment of the sequence is possible by reference to the restriction map so gained.

The insertion of a piece of foreign DNA into an E. coli gene in the correct reading frame allows the expression of a fusion protein, in which part of the amino acid sequence is derived from the E. coli gene and part of it is derived from the inserted DNA. Suitable expression vectors with appropriate control sequences and convenient restriction sites have been constructed, which allow high levels of expression of fusion proteins.

Thus, examination of the restriction map of the expression system of choice and the sequences to be expressed, together with a knowledge of the translational frame, enables specific DNA fragments to be ligated into the expression vector and expressed, without further manipulation. For example, pWRL507 is a plasmid constructed from pAT153 and the trpE gene (Nichols, B.P. et al., J. Mol. biol. 146, 45-54 (1981)) with a synthetic EcoRI-Bgl II linker inserted at the Bgl II site at nucleotide 1223 in the trpE gene (Figure 4). This vector can be cut with Nde I and EcoRI, EcoRI and BamHI, or EcoRI and Hind III and the small DNA fragment(s) replaced with the 2.7 Kbp Nde I-EcoRI fragment of pPFgl, the 400 bp EcoRI-BamHI fragment of pPFc1028 or 2.4 Kbp EcoRI-Hind III (where the Hind III site is in the polylinker of the plasmid) fragment of pPFc1028, respectively. In addition, specific fragments such as the 1.2 Kbp EcoRI-Nde I

fragment of pPFc1028 can be subcloned into the polylinker region of pUC9 (in this instance as an EcoRI/blunt end fragment), then cut out with EcoRI and Hind III and cloned into pWRL507 cut with EcoRI and Hind III.

By using suitable restriction enzyme sites, DNA fragments from the P.195 gene sequence may be cloned into a site within the trpE gene in the correct orientation, but usually in the wrong translational frame. In order to obtain expression of the inserted sequence, a synthetic linker of suitable length can be inserted into the restriction site between the trpE gene and the insert, to give in-frame expression of the fusion protein. Alternatively the plasmid containing the inserted DNA can be opened at the unique restriction site between the bacterial and the inserted DNA and the DNA treated briefly with the enzyme Bal 31 to remove a few bases from each end of the linearised DNA. After repair with the large (Klenow) fragment of DNA polymerase 1, the plasmid is recircularised with T4 ligase and used to transform bacteria. One in three of the transformants should contain the P.195 sequence in the correct reading frame to be expressed as a fusion protein with the trpE gene product. It will be appreciated by those skilled in the art that the extent of digestion with Bal 31 will determine the final size of the expressed fusion protein and the relative lengths of trpE and P.195 sequences contained within it. In addition, the insertion of a synthetic linker during ligation after Bal 31 digestion and repair facilitates the analysis of particular strains after transformation. By the judicious use of specific restriction enzyme digestion and Bal 31 enzyme treatment, any specific region of P.195 can be expressed as a fusion protein.

Thus, in another feature of the invention is provided a vector, containing a DNA sequence according to the invention tandemly linked to an amino-terminal coding portion of a gene translatable by the relevant host and, optionally, any control sequence(s) associated therewith, which, when used to transform a suitable host will result in production of a fusion protein comprising at least a part of P.195 or a peptide comprising at least one epitope thereof.

An alternative method for expressing DNA fragments is to use an open reading frame (ORF) vector into which (usually) short pieces of DNA can be inserted, often within the sequence coding for the N-terminal amino acid sequence of an E. coli protein. The inserted DNA must contain no stop codons in the correct translational frame, be in the correct orientation relative to the direction of transcription, and be in the correct frame at each end. For pieces of DNA from a protein coding sequence generated by a random cleavage method the

theoretical probability of read-through in the correct frame is 1 in 18. ORF vectors based on β-galactosidase have been described (Koenen et al., 1982). Insertion of a piece of DNA into a site at the N-terminus of the protein in the correct frame confers read-through expression of the β galactosidase protein which can be detected by hydrolysis of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal). For example if Xgal is included in the agar upon which the colonies are grown, transformation of a suitable host strain with a plasmid expressing a functional β-galactosidase will produce a blue colony. One such vector, pXY460, contains the β-galactosidase gene under the control of the tac promoter. Insertion of DNA into the Sma I site next to the EcoRI site may convert the gene to in-frame expression. Transformation of an E. coli host such as JM105 converts the bacteria to ampicillin resistance and expression of the fusion protein can be induced at high levels by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG).

Thus, in an alternative aspect of the invention, we provide a vector comprising a gene capable of translation in a suitable host, optionally provided with relevant control sequences, into which is inserted a DNA sequence according to the invention suitably altered such that the portion of said gene carboxy-terminal to said DNA sequence is correctly translated on expression in a suitable host to produce a fusion protein comprising at least a part of P.195 or a peptide comprising at least one epitope thereof and part of said gene-encoded protein.

The peptide coded by part of the P.195 gene in a fusion protein may be cleaved from that fusion protein by enzymic or chemical cleavage of the appropriate peptide bond. It will be apparent to those skilled in the art which enzymic or chemical cleavage method should be employed, by examination of the amino acid sequence expressed. By insertion of a synthetic oligonucleotide linker between the P.195 DNA sequence and the bacterial gene sequence in the fusion protein expression system, a suitable site may be provided for enzymic or chemical cleavage between the P.195 sequence and the remainder of the expressed fusion protein. In this way the P.195 gene-encoded fragments may be purified away from host peptides.

Direct expression of the coding sequence for the P.195 protein or parts thereof can be achieved by placing the inserted DNA sequence directly after an AUG start codon in the correct reading frame such that the DNA insert replaces the

coding sequence normally transcribed and translated by the bacterial control region. Such a control region includes a promoter and a ribosome binding site in the optimal position relative to the start codon. The DNA sequence to be expressed may be correctly positioned by the use of suitable restriction sites and if necessary by using a suitable synthetic oligonucleotide linker. At the end of the inserted DNA sequence a stop codon may be inserted in the correct reading frame to stop translation and a terminator sequence to stop transcription may be added. The inserted DNA to be expressed may be the entire coding sequence of P.195 or the entire sequence from which the amino-terminal signal sequence has been removed, or preferably a part of the coding sequence corresponding to an immunogenic fragment of the protein. Suitable fragments may be prepared by restriction enzyme digestion of a suitable cDNA or genomic DNA clone (after examination of the nucleotide sequence), and if necessary further treatment at either or both ends with Bal 31 to digest away in a controlled way parts of the DNA sequence. Controlled digestion is achieved by the selection of proper buffer, temperature, reaction time and amount of enzyme, for example as described in Example 8. At this stage a suitable synthetic linker may be added, preferably by blunt end ligation to the insert, to provide an AUG start codon or facilitate ligation into the expression vector.

According to a further feature of the present invention we provide a method which comprises transforming a host cell with the above-defined cloning vector and culturing the host cell to provide expression of the said P.195, or a peptide comprising at least one epitope thereof.

Controlled expression of any cloned fragment will be possible by use of the sequences at either end of the sequence, or by use of other sequences already known. Such sequences include promoters and enhancers. Examples of such promoters include lac, trp, bacteriophage λ p_L and hybrid trp-lac(tac). Suitable enhancers include the SV40 enhancer and the enhancer from bovine papillomavirus.

According to a further feature of the present invention we provide a vector containing the above-mentioned DNA sequence according to the present invention.

The vector referred to above may be any appropriate vector which is suitable for the cloning of the DNA and which may be used to transform a host cell and thereby express the relevant protein. Such vectors include plasmids,

bacteriophages and cosmids. Vectors which may be used for cloning cDNA include pUC8, pUC9, pAT153, pBR325 and pBR328 for use in Escherischia coli, pBD9 and pKT438 for use in Bacillus subtilis, pMA56 for use in yeast and pAdD26SV(A)-3, pSV2-dhfr, SVEHA3 and SVLHA8 for use in mammalian cells.

Vectors for use in expression of the relevant protein will include control sequences, such as mentioned above. Such vectors include pXY460 and pWRL 507 for use in E.coli or pSV2-dhfr for use in mammalian cells.

In a further aspect of the invention, we provide a vector containing the DNA sequence according to the invention, further containing one or more control sequences to regulate the expression of said DNA sequence.

Examples of suitable host cells for use in the above-described method may be prokaryotic, such as bacteria (for example E.coli HB101 and DH1, B. subtilis sp.BD170 and IH6140), or eukaryotic, such as yeast (for example XV610-8C yeast cells) or mammalian cells (for example simian CV-1 cells).

In an alternative embodiment of the invention we provide a method for synthesising at least a portion of P.195 or a peptide comprising at least one epitope thereof, said P.195 or peptide being optionally covalently linked to further peptide sequence, which method comprises the steps of:

a) creating a cDNA or genomic DNA library from Plasmodium falciparum;
b) selecting a probe for P.195 and rendering the said probe radio-active;
c) selecting a member or members of the said library by use of the said probe; and
d) using DNA thus selected from the said library to transform a suitable host which may be used to express the said P.195 or a peptide comprising at least one epitope thereof.

The present invention further includes the said P.195 protein or peptides comprising at least one epitope thereof, when obtained by any of the above methods according to the invention. These materials may be incorporated into a vaccine for conferring immunity against malaria. For this purpose the antigenic protein or a peptide comprising at least one epitope thereof may be presented in association with a pharmaceutically acceptable carrier. The antigenic proteins or peptides may be used singly or in combination with other

P.195 epitope-containing peptides or with other proteins which will provide immunity against malaria.

In a further aspect there is provided a vaccine for inducing immunity to malaria which comprises P.195 or a peptide comprising at least one epitope thereof, in association with a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers, in this instance, are liquid media suitable for use as vehicles to introduce the antigen into the patient. An example of such a carrier is saline solution. The P.195 or peptide may be in solution or suspended as a solid in the carrier, or it may be solubilised by the addition of pharmaceutically acceptable detergent.

The vaccine may also comprise an adjuvant for stimulating the immune response and thereby enhancing the effect of the vaccine. A convenient adjuvant for use in the present invention is aluminium hydroxide.

Conveniently the vaccines are formulated to contain a final concentration of P.195 or peptide in the range of from 0.2 to 5 mg/ml, preferably 0.5 to 2 mg/ml, most preferably 1 mg/ml. After formulation the vaccine may be incorporated into a sterile container which is then sealed and stored at a low temperature, for example 4°C, or it may be freeze-dried.

In order to induce immunity in vertebrate hosts to malaria one or more doses of the vaccine suitably formulated may be administered. It is recommended that each dose is 0.1 to 2 ml preferably 0.2 to 1 ml, most preferably 0.5 ml of vaccine.

There is in a further aspect provided a method for inducing immunity to malaria in susceptible vertebrate hosts, comprising the administration of an effective amount of a vaccine, as hereinbefore defined, to the host.

The vaccines may be administered by any conventional method for the administration of vaccines including oral and parenteral (eg. subcutaneous or intramuscular) injection. The treatment may consist of a single dose of vaccine or a plurality of doses over a period of time.

The following examples are for illustration only and are not intended to limit the invention in any way.

Example 1

Identification of a cDNA Clone from the P.195 Gene

P.falciparum cultures were maintained and synchronized as described by Holder and Freeman (1982 supra) and cells collected by centrifugation 30-40h. after the last cycle of reinvasion. After washing in PBS (150 mM NaCl, 5mm KCl and 10mM sodium phosphate pH7.2) the cells were resuspended in four volumes of 50mM sodium acetate pH5.5, 100mM NaCl, 1mM EDTA and SDS added to 3% w/v. Vigorous extraction with phenol-chloroform (1:1) equilibrated with the same buffer was performed for 5 minutes followed by centrifugation at 16,000g for 3 minutes. After a second extraction of the aqueous phase, nucleic acids w re precipitated with ethanol, centrifuged, the pellet dissolved in 4ml 0.1M EDTA pH 7.5 and 4g CsCl added. The RNA was pelleted through a cushion of 95% (w/v) CsCl in 0.1M EDTA by centrifugation at 150,000g for 16 hours at 25°C, redissolved in distilled water and precipitated twice with ethanol.

To purify the mRNA, oligo-dT cellulose chromatography was performed by standard methods (Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982), molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, New York). Size fractionation of RNA in sucrose gradients was as described previously (Odink, K.G. et al., J. Biol. Chem. (1981) 256, 1453-1458). A cDNA library was constructed using standard procedures (Maniatis et al., (1982) supra). cDNA was synthesized in a 50 µl reaction containing 6µg Poly A+ RNA, 5µg oligo-$dT_{(12-18)}$, 1mM of each nucleoside triphosphate, 0.1M Tris-HCl pH 8.3, 10mM $MgCl_2$, 140mM KCl, 10 mM DTT and 30 U of AMV reverse transcriptase for 90 min at 42°C.

Second strand synthesis was in 0.1 ml 0.1M HEPES pH6.9, 10mM $MgCl_2$, 2.5mM DTT, 70mM KCl, 0.5mM of each nucleoside triphosphate and 50 U of E.coli DNA polymerase large fragment for 16 hours at 15°C. After S1 nuclease digestion, 5µg of DNA were recovered and 0.5µg inserted into the Pst I site of pUC8 (Vieira J. and Messing, J. Gene 19, 259-268 [1982]) by homopolymeric G-C tailing. E.coli HB101 was used for transformation. Replica filters carrying 3,000 recombinants were probed with γ-^{32}P-ATP polynucleotide kinase-labelled 33S mRNA previously shown by in vitro translation to be enriched for mRNA encoding P.195 (Odink et al, 1984). 60 recombinant plasmids were detected with the probe and 12 of these gave a strong signal. On the basis of cross-hybridization of nick-translated inserts, 6 sub-groups were formed from the 12

recombinants. On the premise that these recombinant probes represented parts of the DNA sequence for P.195, they should hybridize to a mRNA of not less than 5,300 bases long from a total extract of P.falciparum mRNA, this being the minimum estimated length necessary to code for a protein of about 195,000 MW. One member of each group was labelled by nick-translation and used as a probe on P. falciparum RNA Northern blots. (Thomas, P.S. (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 5201-5). Three recombinants, pFC15, pFC16 and pFC17 with cDNA inserts of 1.6kb, 2.3kb and 1.1kb respectively, hybridized to mRNAs of 9kb, 7.5kb and 5.5kb in size respectively. Isolated insert DNA from pFC17 was treated with exonuclease Bal31 to give randomized reading frames and inserted into an expression plasmid carrying part of the tryptophan operon. DNA was inserted into the Bssh II Site, 13 amino acids from the carboxy terminus of the mature trpE-gene product, anthranilate synthetase I. In-phase insertion of cDNA into this site gives a fusion protein carrying 56,000 MW of anthranilate synthetase I. Upon induction of the gene, by tryptophan starvation in the presence of ß-indole acrylic acid, one of the resulting recombinants, pFT 1733, gave a fusion protein of 72,000 MW (as determined by SDS-PAGE) representing an additional 16,000 MW encoded by the cDNA insert.

Bacterial extracts and an extract of P. falciparum schizonts were subjected to SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose and then probed with a polyvalent rabbit serum specific for P.195. The fusion protein of pFT1733 was clearly detected by the antiserum. The antiserum was highly specific for P.195 as, from a total P. falciparum schizont extract, only P.195 was detected. There was no reaction with a bacterial extract containing an 80,000 MW fusion protein consisting of the trpE gene product and a foot and mouth disease virus VP1 protein. In addition, the control using normal rabbit serum showed no binding. Thus, pFC17 encodes some of the antigenic determinants of P. falciparum P.195. Further reference to pFC17 will be by its synonym, pPFc1017.

Example 2

Preparation of Further Overlapping cDNA Clones Related to pPFc1017.

A recombinant cDNA library was constructed from size fractionated cDNA. cDNA, prepared and tailed as described in Example 1, was centrifuged through a 5 ml 5% to 20% (w/v) sucrose gradient in 25mM Tris HCl pH 7.4, 100 mM NaCl, 2.5mM EDTA for 3.5 hrs at 45,000 rpm in an SW50.1 rotor (Beckman Instruments). cDNA in the 2 kbp to 8 kbp region (about 100 ng) was harvested, annealed with 300 ng dG-tailed Pst 1 digested pUC9 and used to transform DH1 cells to ampicillin resistance. 1200 recombinants were obtained on 6 agar plates. Replica filters were probed with the insert DNA from pPFc1017 which had been labelled by nick translation. Eleven clones were indentified as containing DNA hybridising to this probe. These clones were numbered pPFc1001 to 1011. This library was subsequently rescreened using a part of the insert in pPFc1007 which did not cross-hybridise with pPFc1017. A further 8 clones, pPFc1028 to 1035, were isolated which hybridised to this probe.

A further cDNA library was constructed using 100 ng dC-tailed cDNA, annealed with 100 ng G-tailed pUC9 and transformed into DH1 cells. About 6000 recombinants were obtained and these were screened on replica filters with the 340 bp Hind III-Pst 1 fragment from pPFc1017. Clones pPFc1013 to 1016 and 1018 to 1027 which hybridised to this probe were picked and purified. This library was also screened with the pPFc1007 derived probe and a further 11 colonies numbered pPFc1036 to 1046 were picked.

Plasmid DNA from these cDNA clones was purified by centrifugation on caesium chloride gradients and characterized by restriction enzyme mapping and cross-hybridisation. The cDNA clones were aligned in an overlapping linear sequence. The positions of the six cDNA clones used in the sequence analysis are shown in Figure 2.

Example 3

Isolation of Genomic Clone pPFg1 from the P.195 gene.

100 µg P. falciparum DNA (prepared according to the method of Odink et al., (1984)supra) was digested to completion with Hind III, the sample loaded on a 10-40% sucrose gradient containing 1M NaCl, 20 mM Tris. HCl, pH 8.0, 5mM EDTA and centrifuged in a Beckman SW27 rotor for 24 hrs at 26,000 rpm, 20°C. 0.5 ml fractions were collected by bottom puncture of the tube and aliquots of alternate fractions were run on a 1% agarose gel. Fractions in the region of the gradient giving a positive signal on dot blot hybridization with nick-translated pPFc1017 were digested with EcoRI and ligated with gel-purified Hind III + EcoRI cleaved pUC8 DNA. About 400 transformants in DHI from each fraction were screened on nitrocellulose filters by colony hybridisation with pPFc1017 insert (Grunstein, M. and Hogness, D., Proc. Natl. Acad. Sci. 72, 3961 (1975)). One colony from fraction 39 gave a positive signal. This recombinant, pPFg1, contains a 3.1 kb Hind III-EcoRI fragment which co-migrates with the corresponding genomic DNA fragment, and both have the same restriction enzyme maps. A restriction map of $pPFg_1$ was obtained by partial end-label mapping of pPFg1 insert (Smith, H.O. and Birnstiel, M. L., Nucl. Acid Res. 3, 2387 (1976)). Sequencing was performed by the Sanger dideoxy method described in Example 5.

Example 4

Construction of a Map of the P.195 Gene in P. falciparum DNA

P. falciparum DNA was prepared as described (Odink et al., (1984) supra) and aliquots of it were restricted with specific endonucleases, either individually or on some occasions as a double digest, i.e. with two restriction endonucleases. The products were electrophoresed on agarose gels (0.7% to 1.5%) in Tris-Borate-EDTA (pH 8.2) containing 0.5 µg/ml Ethidium bromide and together with DNA fragments of known length as size markers in parallel tracks. The DNA was transferred to Gene Screen Plus (New England Nuclear/Dupont) by a capillary blot procedure and hybridised to ^{32}P-labelled probe DNA at 42°C in the presence of 50% formamide, according to the protocols suggested by the manufacturer. Hybridised probe DNA was detected by autoradiography at -70°C using X-Omat S film between Cronex Lightning-Plus screens.

The probe DNA was specific plasmid DNA or specific sequences excised from the cDNA or genomic DNA plasmid clones, and purified by agarose gel electrophoresis and elution. The DNA was labelled with ^{32}P by nick translation with E. coli DNA polymerase in the presence of ^{32}[P] α-ATP. An analysis of the size of restriction fragments from within the genomic DNA hybridising to specific probes enabled a linear map of restriction enzyme sites to be constructed. Such a map of the P.195 gene is shown in Fig.3 with some exemplary sites and probes to which the specific fragments hybridised. It will be appreciated that several other specific digests of genomic DNA were performed and were probed with other specific fragments from the cloned DNA, and the results were consistent with the map shown in Fig.3. To improve clarity of the map, not all of the restriction sites present in the DNA sequence are indicated.

An examination of the genomic map and the restriction enzyme map derived from the DNA sequence indicates that these two are colinear in the region corresponding to the coding sequence for the protein, to the right hand side of the Hind III site at nucleotide 313. However, there appears to be an additional 700 bp of sequence in the genomic DNA, not present in the cDNA clones, located between the Mbo 1 site and the Hind III sites at nucleotides 221 and 313 in the cDNA sequence. This may represent an intron in th very start of the coding sequence.

The sites shown on the map are some of those for the enzymes A (AluI), B(Bam HI), E(EcoR1), H(Hind III), M(MboI), N(NdeI), P(PstI), Pv(PvuII), R(RsaI) and T(TaqI). The probes were either total plasmids or specific fragments derived by digestion with specific enzymes at sites within the insert or within the plasmid polylinker region.

Example 5

Nucleotide Sequence of the DNA Coding for P.195

Sequence analysis of the DNA was done using the chemical cleavage method of Maxam and Gilbert (Maxam and Gilbert, 1980, supra) and the dideoxy procedure of Sanger (Sanger et al., 1977, supra).

1. Chemical cleavage

The DNA fragments suitable for sequence analysis were prepared as follows.

a) The DNA was digested with restriction endonuclease (under the conditions described by the suppliers), then calf intestine alkaline phosphatase (Boehringer Mannheim) was added to the mixture and the reaction continued for 30 min. at 37°C. The DNA was extracted with chloropane and ethanol precipitated. The 5' ends of the DNA were labelled with [^{32}P] using polynucleotide kinase as described by Maniatis (Maniatis et al., 1982, supra). The labelled DNA was cut with a second suitable restriction endonuclease and the mixture was loaded onto 1% (w/v) agarose gel, the DNA bands of interest were electro-eluted from this agarose gel and their sequences were determined.

b) Fragments of DNA were also prepared by modification of the procedure described in section a). The DNA was digested with restriction endonuclease, phosphatased and labelled at the 5' end with [^{32}P] as outlined above. The DNA fragments were then denatured by addition of de-ionized formamide (final concentration 70% (v/v)), and heated to 100°C for 5 minutes. The samples were quickly cooled in iced water and immediately loaded onto a non-denaturing 15% polyacrylamide gel (with acrylamide to bis-acrylamide ratio of 60:1 (w/w)). The separated DNA strands were electro-eluted from the gel and sequenced.

2. Dideoxy sequencing

The DNA templates were prepared by subcloning fragments of the insert in the filamentous phage cloning/sequencing vector M13mp8 (Messing and Vieira, 1982, supra). The sequencing was performed using a synthetic universal primer (Celltech) and [^{35}S]-d ATPαS (Amersham International) as described by Sanger et al., 1977, supra. Two basic strategies were used to sequence specific

fragments. In the first, specific restriction fragments (produced by digestion with RsaI, HinfI, RsaI-Aha III, TaqI) were purified by electro-elution, and where necessary, the staggered ends made blunt using the Klenow DNA polymerase I fragment. Fragments difficult to clone or sequence by the above protocol were treated with Bal-31 (Maniatis et al., 1982). Conditions (DNA and enzyme concentration) were chosen such that 100-150 bp DNA was removed from each end of the fragment in 1 minute at 30°C. By digesting over a time course a series of overlapping fragments were obtained. Bal-31 treated DNA was repaired with DNA polymerase I Klenow fragment. DNA was ligated with phosphatase-treated SmaI-digested M13mp8 (Amersham) and transfected into JM103 or JM101 (Messing, J., et al., Nucl. Acid Res. 9, 309 (1981)). Template DNA was prepared according to standard procedures. Wherever possible, sequence was obtained from both strands of each clone.

The total sequence obtained by overlapping the sequences of the individual clones is shown in Figure 1. The clones used to determine the sequence are shown beneath the restriction map obtained from the DNA sequence in Figure 2.

Example 6

Purification of the 83,000 MW Fragment from Culture Supernatants and the Determination of a Partial Amino Acid Sequence

Supernatants from in vitro cultured P. falciparum (described in Example 1) were harvested and centrifuged at 10,000g for 5 minutes to remove cellular debris. To each 100ml culture supernatant was added 1ml 1M Tris, 100 mM EDTA, 100 mM EGTA, 1 ml 100 mM PMSF, 1 ml 0.5M iodoacetamide, 1 ml 10 mM TLCK and 0.5g sodium deoxycholate. The pH was adjusted to 8.2 with HCl and then the sample was centrifuged at 100,000g for 45 minutes. The supernatant after centrifugation was then applied to a 10 ml column of antibody 89.1-Sepharose (prepared as described by Holder and Freeman, 1984b supra) which had been pre-equilibrated with 10 mM Tris-HCl pH 8.2 containing 1mM EDTA; 1mM EGTA and 0.5% (w/v) sodium deoxycholate (equilibration buffer). After the column had been washed extensively with equilibration buffer, material retained on the column was eluted with 50 mM diethylamine HCl, pH 11.5, containing 0.5% (w/v) sodium deoxycholate. The eluate was concentrated by ultrafiltration using an Amicon XM50 filter and adjusted to pH 8.2. The major polypeptide in the eluate was the 83,000 MW species, and it was the only component which reacted with either monoclonal antibody 89.1, rabbit polyvalent anti-P.195 serum or human P. falciparum immune serum, on Western blots. The major contaminant in the preparation was IgG which was removed from the concentrated eluate by passage through a Protein A-Sepharose column (0.9 x 10 cm) (Pharmacia Fine Chemicals) equilibrated as described above. The unretarded material, depleted of IgG, was collected. Solid guanidinium chloride was added until a clear solution was obtained and then the protein was reduced and S-carboxymethylated (Waxdal M.J. et al.,Biochemistry 7 1959-1966 (1968)). The reduced and S-carboxymethylated 83,000 MW polypeptide was finally purified by passage through a column of Sephacryl S300 (Pharmacia Fine Chemicals, Sweden) equilibrated with 10 mM Tris HCl pH 8.2, containing 1 mM EDTA, 1mM EGTA and 6M guanidinium chloride. Fractions were assayed spectrophotometrically at 280 nm and by SDS-PAGE analysis of aliquots, and those containing the 83,000 MW species were pooled. After extensive dialysis against water and 5% (v/v) formic acid the sample was freeze-dried, prior to application to the automatic sequencer in a small volume of 5% formic acid. The protein was subjected to 20 cycles of automated Edman degradation in a Beckman 890C sequencer equipped with a Sequamat P6 autoconverter and a Sequamat SC-510 program controller using a 0.33M Quadrol program, as

described by Baccanari D.P. et al.,J. Biol. Chem. 259, 12291-12298 (1984). The released phenylthiohydantoin derivatives of amino acids were identified by reverse-phase HPLC and confirmed by back hydrolysis (Baccanari et al., 1984 supra). Starting from 910 ml of culture supernatant, 5.9 mg of protein was present in the eluate from the affinity column, 2.6 mg of protein passed through the protein A-Sepharose column and 400µg of purified protein was subjected to the Edman degradation.

The following partial amino acid sequence was obtained from the successive cycles of degradation starting at the N-terminal amino acid of the polypeptide.

1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
N.I.	N.I.	N.I.	N.I.	N.I.	Tyr	Gln	Glu	Leu	Val
11.	12.	13.	14.	15.	16.	17.	18.	19.	20.
Lys/Phe	Lys/Phe	Leu	Glu	Ala	Leu	Glu	Asp	Ala	Val

The residues at positions 1 to 5 were not clearly identified (N.I.) and lysine and phenylalanine derivatives were not separated from each other in the HPLC system employed.

By examination of the nucleotide sequence derived from the cDNA and genomic DNA clones it can be seen that this sequence of residues (6 to 20) corresponds to the translated sequence of nucleotides 288 to 332.

On this analysis the 83,000 MW fragment is derived from the amino terminal sequence of the P.195 precursor. In the complete sequence the AUG (methionine) start codon at nucleotide 216 is followed by nucleotide sequence of 18 amino acids which may correspond to a signal sequence present on the primary translation product for many membrane or secreted proteins, and which is normally cleaved off during the passage of the protein into the lumen of the endoplasmic reticulum (Kreil, G., Ann. Rev. Biochem.50, 317-348 (1981)).

Example 7

The Reaction of Antibodies with Processing Fragments of P.195 and the Position of these Fragments within the Linear Coding Sequence.

By direct amino acid sequencing, the amino-terminus of the 83,000 MW fragment has been located close to the amino terminus of P.195 (Example 6). The other fragments produced by in vivo proteolytic processing can be located in the linear gene sequence by an analysis of the sizes of polypeptides recognised by specific antibodies (preferably monoclonal antibodies) raised against P.195. Two monoclonal antibodies were used in this analysis. Antibody 89.1 (Holder and Freeman, 1982, supra) reacts with intracellular P.195 in schizonts and with the 83,000 MW fragment on the merozoite surface. It does not react (by immunofluorescence) with the subsequent ring stage of the parasite, consistent with the loss of this fragment during merozoite invasion of red cells. Antibody 111.2 reacts with intracellular P.195 in schizonts, with the merozoite surface and with the ring stage parasite by immunofluorescence. This specificity is similar to that of a monoclonal antibody described recently by Hall et al. (1984a, supra).

P.195 and processing fragments of it can be labelled in vitro by the addition of [^{35}S] methionine to cultures of the parasite in methionine-free medium (Holder and Freeman, 1982, supra, Freeman and Holder, 1983, supra). The processing fragments on the surface of naturally released merozoites can be labelled by radio-iodination with [^{125}I] iodine using lactoperoxidase as described (Freeman and Holder, 1983b supra; Holder and Freeman 1984b, supra).

From detergent extracts of [^{35}S] methionine-labelled schizonts a rabbit polyvalent antiserum against P.195 immunoprecipitated P.195 and fragments which have molecular weights of 153,000; 110,000; 83,000; 45,000; 42,000 and 29,000 as determined by SDS-PAGE analysis. On non-reducing gels (in the absence of dithiothreitol) the 45,000 and 42,000 MW species migrated with apparent molecular weights of 38,000 and 36,000 respectively. Antibody 89.1 immunoprecipitated P.195 and the 153,000, 110,000 and 83,000 MW species. Antibody 111.2 immunoprecipitated P.195 and the 45,000 and 42,000 MW species.

From detergent extracts of surface-labelled merozoites the polyvalent anti-P.195 serum immunoprecipitated three fragments which have apparent molecular weights of 83,000, 42,000 and 19,000. The 83,000 MW fragment was

immunoprecipitated by antibody 89.1. Antibody 111.2 immunoprecipitated the 42,000 and 19,000 MW species. An analysis of these fragments by peptide mapping showed that they were probably non-overlapping pieces of P.195 (Holder and Freeman, 1984b supra).

Based on these data and that described in examples 6 and 10 the linear order of the fragments within the P.195 coding sequence can be determined. The amino acid sequence determined for the amino terminus of the 83,000 MW fragment corresponds to the sequence at the 5' end of the coding region of the gene, immediately after the presumptive signal sequence. Therefore the 83,000 MW fragment is derived from the amino terminal 42% of P.195. The major intermediate processing fragment of 153,000 MW which is recognised by antibody 89.1 and not by antibody 111.2 is derived from the amino terminal 78.5% of the coding sequence, and therefore including all of the 83,000 MW fragment. The 42,000 MW fragment is derived from the carboxy-terminal 21,5% of the coding sequence, since the antibody 111.2 does not react with the 153,000 MW fragment. The 19,000 MW fragment is presumably located within the sequence of the 153,000 MW species, but not within the 83,000 MW species.

Example 8

Expression of P195 Sequences as Fusion Proteins in E. coli and Purification of the Products

In separate experiments, pWRL507 (Fig.4 of the accompanying drawings) was digested with Ndel and EcoR1, EcoR1 and BamH1 or EcoR1 and Hind III and the relevant fragments purified by agarose gel electrophoresis. This DNA (0.1 pmoles) was ligated with 0.5 pmoles of the DNA fragments derived from the P.195 recombinants by the relevant pairs of restriction enzymes and then used to transform DH1 cells to ampicillin resistance. Colonies were screened by restriction enzyme digests of small plasmid preparations. Strains with plasmid containing the insert were further grown in M9 minimal medium containing 100 µg/ml ampicillin and 10 µg/ml indole acrylic acid (induced expression) or 100 µg/ml ampicillin and 10 µg/ml tryptophan (non-induced). Bacteria were harvested by centrifugation at 10,000g for 1 minute and then lysed by the addition of SDS sample loading buffer for PAGE (62.5 mM Tris-HCl pH 6.8 containing 2% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol, 0.1M dithiothreitol and 0.005% (w/v) bromophenol blue). Aliquots were subjected to analysis by SDS-PAGE and then the gels were stained with coomassie blue to detect total protein or the resolved proteins were transferred to nitrocellulose and used for Western blotting with a polyvalent antiserum raised against purified P.195. A strain containing the Nde 1 - EcoR1 fragment of pPFg1 produced an inducible fusion protein of 135,000 MW which could be detected in lysates by coomassie blue staining and by reaction with the polyvalent antiserum. This corresponds to a fusion protein containing 233 amino acids from the N-terminus of the trpE gene product and 907 amino acids from P.195. A strain containing the EcoRI-BamH1 fragment of pPFc1028 produced a fusion protein of 53000 molecular weight containing 326 amino acids from trpE and linker and 156 amino acids from P.195. A strain containing the EcoRI-Hind III fragment of pPFc1028 produced a fusion protein of 105,000 MW consisting of 326 amino acids from trpE and linker and 594 amino acids from P.195. A strain containing the EcoRI-Nde I fragment from pPFc1028 produced a fusion protein of 85,000 MW consisting of 326 amino acids from trpE and 401 amino acids from P.195. In each case the fusion protein was detected by coomassie blue staining and reacted with the polyvalent anti-P.195 serum on Western blots.

A 750 bp Rsa I Hind III fragment derived from the cDNA clone pPFc1013, comprising nucleotides 863 to 1613 together with a G-C tail and part of the pUC9 polylinker region from the Pst I site to the Hind III site was sub-cloned into the plasmid pUC9 which had been cut with Hind II, treated with calf intestinal phosphatase and further digested with Hind III. The insert was subsequently cut out of pUC9 using EcoRI and Hind III and inserted (in the correct orientation) into pWRL507 cut with EcoRI and Hind III. To obtain in-frame expression, 5µg of the plasmid was treated with EcoRI restriction enzyme to linearise the construct. The DNA was precipitated with ethanol, re-dissolved in 50 µl H_2O containing 500 µg/ml BSA and then mixed with an equal volume of Bal 31 buffer (Maniatis et al., (1982) supra). The DNA was digested with 0.02 units of enzyme Bal 31 (Biolabs) for 1 minute at 30°C and then the reaction was terminated by the addition of 20 ul 1M Tris 100mM EDTA 100mM EGTA. The DNA was purified by agarose gel electrophoresis, and then treated with DNA polymerase 1 large fragment (Klenow) in the presence of nucleotide triphosphates using 2.5 units of enzyme (Boehringer/Mannheim) in a final volume of 50 µl nick translation buffer (Maniatis et al., (1982) supra) for 90 minutes at room temperature. The DNA (0.1 pmole) was then recircularised by incubation overnight with 200 units T4 DNA ligase (Biolabs) at 7°C, and then used to transform DH1 cells to ampicillin resistance. Ten individual transformed strains were screened by restriction enzyme analysis of the plasmid DNA. One group of 8 transformants contained plasmids which had lost the EcoRI site. This group was further analysed by growth in M9 medium in the presence of indole acrylic acid or tryptophan. One strain produced a fusion protein of approximately 65,000 MW when grown in the presence of indole acrylic acid, and this fusion protein reacted with polyvalent anti-P.195 serum on a Western blot.

In a similar manner the Nde I-Hind III fragment from pPFc1028 (where the Hind III site is the plasmid polylinker) was first subcloned as a blunt end-Hind III fragment into pUC9 that had been cut with Hind II and Hind III. After inserting this fragment into pWRL507 cut with EcoRI and Hind III, the new construct was reopened with EcoRI, treated with Bal 31 and then recircularised as described above. One strain produced a fusion protein of approximately 56,000 MW which was detected on Western blots with anti-P.195 serum, and containing the C-terminal 190 amino acid sequence of the coding region for the P.195 gene.

Relatively pure preparations of the individual fusion protein were produced from cell lysates. A single colony from a strain was grown up overnight at

37°C in 100 ml M9 medium containing 50 µg/ml ampicillin. The following day the overnight culture was diluted with 400 ml M9 culture medium containing 50 µg/ml ampicillin and 10 µg/ml indole acrylic acid and incubated for 5 hrs at 37°C. The bacteria were harvested by centrifugation at 6000g for 10 minutes. The bacterial pellet was suspended in 10 ml 25mM Tris pH 8.0 containing 1mM EDTA, 1 mM PMSF, 0.2% (v/v) NP40 and 1mg/ml lysozyme and left on ice for 2 hrs. After this time 20 µl of 1M $MgSO_4$ and 200 µl of 1 mg/ml DNAse were added and the sample was left to incubate for a further 2 hrs on ice. The insoluble material was harvested by centrifugation at 20,000g for 10 minutes and the supernatant (S1) was retained. The pelleted material was washed by suspension in 10 ml 50mM Tris-HCl pH 8.0 containing 5mM EGTA, 5mM EDTA, 1 mM PMSF and 1% NP40. The material was centrifuged at 20,000g for 10 minutes and the supernatant was retained (S2). The pellet was resuspended in 10ml 50mM Tris HCl pH 8.1, 5mM EGTA, 5mM EDTA, 0.5M KSCN, and then centrifuged at 20,000g for 10 min to yield a third supernatant (S3) and a pellet fraction (P). The pellet fraction was resuspended in 10ml H_2O with and without the addition of 0.1% (w/v) sodium dodecyl sulphate, to solubilise the material and then dialysed extensively against 0.89% NaCl. Aliquots of each supernatant and pellet fraction were analysed by SDS-PAGE and coomassie blue staining and by Western blotting. In instances where there was a high level of expression of fusion protein, this procedure resulted in a final pellet fraction that contained predominantly the fusion protein and therefore constituted an effective purification of the fusion protein. In instances where the level of expression was lower, the fusion protein was present in both S1 and P fractions.

Two Dde 1 Fragments from pPFc1028 corresponding to nucleotides 2961 to 4754 (1793 nucleotides) and nucleotides 4753 to 5128 (375 nucleotides) were purified by agarose gel elctrophoresis, treated with 0.04 units of Bal 31 nuclease at 30°C for 2.5 minutes and then repaired with the Klenow Fragment of DNA polymerase I as described above. The DNA was ligated into PXY460 which had been cut with Sma 1 and treated with calf alkaline phosphatase and this was then used to transform JM105 cells to ampicillin resistance. Transformants were plated onto Agar plates containing Xgal and resultant blue colonies were picked. Strains obtained in this way were screened by restriction enzyme analysis of the plasmid DNA,coomassie blue staining and Western blotting of lysates from cells grown in the presence of IPTG. Strains containing the large Dde1 fragment all produced a large fusion protein which reacted with the rabbit polyvalent anti-P.195 serum. 10 strains containing the smaller Dde1 fragment were investigated as described but only one appeared to produce a fusion

protein which was significantly larger than wild type β-galactosidase and this fusion protein reacted with the rabbit anti-P.195 serum. Although the remaining strains contained the insert the gene product was the same size as the normal β-galactosidase and did not react with the rabbit anti-P.195 serum. The one strain that produced a fusion protein contained an insert of 310bp which covers the C-terminal region of the protein.

Example 9
Direct Expression of P.195 Sequences in E. coli

The EcoRI-Nde I and EcoRI-Hind III fragments derived from pPFc1028 were inserted into pWRL507 as described in Example 8, and were expressed directly by replacing the Pst I-EcoRI fragment in the trpE expression plasmid by the Pst I-EcoRI fragment from pXY460 (Figure 4). In this construction the trp control region and coding sequence are replaced by the tac control region (de Boer, H.A. et al., Proc. Natl. Acad. Sci 80, 21-25 (1983)) and an AUG start codon. Directly expressed products were detected in cell lysates by Western blotting with antisera raised against purified P.195. The direct expression products from these two constructions had apparent molecular weights of 47,000 and 70,000 respectively.

Example 10

Analysis of the Immunogenicity and Antigenicity of the Fusion Proteins Produced in E. Coli

A rabbit polyvalent antiserum specific for the P. falciparum derived P.195 was obtained by immunising a rabbit with 100 µg of the protein in Freund's Complete Adjuvant (FCA, Difco Laboratories, Detroit) and then boosted with 100 µg of the protein in Freund's incomplete adjuvant (FIA) on days 22, 43 and 211. On day 218 after the primary immunisation the serum was collected.

Polyvalent antiserum was produced by immunization with the purified fusion proteins (Example 8). Rabbits were immunized subcutaneously with 250 µg of the protein in FCA and then boosted with 250 µg of the protein in FIA on day 21. On day 35 after the primary immunization serum samples were collected. Mice were immunized intraperitoneally with 125 µg of the protein in FCA and boosted with the same dose on day 23. On day 30 after the primary immunisation serum samples were collected.

The titration of an antiserum for the binding of antibodies to a protein can be quantified by a solid phase radioimmunoassay (RIA) in which the wells of a microtitre plate are coated with the protein and then serial dilutions of the antibody solution are added to the series of wells. After washing away the unbound antibodies the bound antibodies are detected using a highly labelled specific reagent for the first antibodies, such as protein A from Staphylococcus aureus of affinity purified IgG specific for the first antibodies. The proteins which were used to coat the microtitre plates were either the fusion proteins purified from lysates of E. coli as described in Example 8, or the P.195 purified by monoclonal antibody affinity chromatography from extracts of P. falciparum infected red cells as described by Holder and Freeman (1984b, supra).

The antigens were diluted to 20 µg/ml in 0.05M $NaHCO_3$ pH 9.6 and 50 µl of this solution was added to each well of a 96 well PVC microtitre plate (Dynatech Laboratories). After 90 minutes the plate was washed thoroughly with phosphate-buffered saline supplemented with 0.5% (v/v) Tween 40 and 0.2% (w/v) bovine serum albumin (wash buffer). 50 µl of each serum dilution was added to duplicate wells and after 30 minutes the plate was washed for 10 minutes in wash buffer. Specific antibody was detected by addition of 50 µl of

^{125}I-labelled protein A (1.5 x 10^5 cpm) for 30 minutes, followed by extensive washing and determination of bound radioactivity using a Packard PED gamma counter.

As can be seen from Table 1 rabbits immunized with the fusion proteins containing P.195 sequences produced antibodies which reacted with the purified P.195 in the RIA and the polyvalent antiserum against P.195 contained antibodies which reacted with the fusion proteins. In this instance, fusion protein 1 is the product of the pPFc1028 EcoRI-NdeI fragment inserted into trpE and fusion protein 2 is the product of the pPFc1028 EcoRI-Hind III fragment inserted into trpE.

In Example 8 it was shown that the fusion proteins reacted with antibodies in the polyvalent anti-P.195 antiserum on Western blots. The antiserum raised against the fusion proteins reacted with the fusion proteins and with the purified P.195 protein, on Western blots.

The position of the processing fragments in the linear coding sequence was determined partially by immunoprecipitation from a detergent extract of ^{35}S-methionine labelled P. falciparum (Example 7). When the antisera against the fusion proteins were used to immunoprecipitate proteins from this extract, P.195 was recognised in each case. In addition the rabbit antiserum raised against protein coded by the pPFc1028 EcoRI-NdeI insert reacted predominantly with the 153,000 and 29,000 MW fragments. The rabbit antiserum raised against the pPFc1028 EcoRI-Hind III insert reacted predominantly with the 42,000 MW fragment.

Table 1 Antibody Binding to Different Antigens by Solid Phase RIA 0154454

^{125}I-Protein A (cpm) bound by antibodies in antisera at $\frac{1}{10}$ dilution

Antigen	Normal Rabbit	Rabbit anti-P.195	Rabbit anti-Fusion Protein 1	Rabbit anti-Fusion Protein 2
P.195	161	6581	1959	1432
Fusion Protein 1	234	2007	10952	13291
Fusion Protein 2	52	1093	3600	3416

Claims

1. A cloned DNA sequence substantially encoding the P.195 protein of P. falciparum or a peptide comprising at least one epitope thereof.

2. A DNA sequence according to claim 1 comprising at least a part of the DNA sequence shown in Figure 1 of the accompanying drawings.

3. A DNA sequence according to claim 1 possessing substantially the restriction sites as shown in Figures 2 and 3 of the accompanying drawings.

4. A DNA sequence according to claim 1 substantially encoding any one of the P.195 fragments occurring in vivo.

5. A cloned DNA sequence substantially encoding the naturally occurring 42,000 MW fragment of P.195 or a peptide comprising at least one epitope thereof.

6. A P.195 protein or a peptide derived from a DNA sequence according to any of claims 1 to 5 comprising at least a part of the amino acid sequence as shown in Figure 1 of the accompanying drawings.

7. A vector containing a DNA sequence according to any of claims 1 to 5.

8. A vector containing a DNA sequence according to any of claims 1 to 5 tandemly linked to an amino-terminal coding portion of a gene translatable by a suitable host which, when used to transform a suitable host will result in production of a fusion protein comprising at least a part of P.195 or a peptide comprising at least one epitope thereof.

9. A vector according to claim 8 further comprising at least one control sequence.

10. A vector according to claim 7 or claim 8 further containing at least one control sequence to regulate the expression of said DNA sequence in a suitable host.

11. A vector comprising a gene capable of translation in a suitable host into which is inserted a DNA sequence according to any of claims 1 to 5 suitably altered such that the portion of said gene carboxy-terminal to said DNA sequence is correctly translated on expression in a suitable host to form a fusion protein comprising at least a part of P.195 or a peptide comprising at least one epitope thereof and at least a part of said gene-encoded protein.

12. A vector according to claim 11 further comprising at least one control sequence.

13. A method comprising transforming a host cell with a vector according to any of claims 7 to 12 and culturing the host cell to provide expression of the said P.195, or a peptide comprising at least one epitope thereof.

14. A cell containing a vector according to any of claims 7 to 12.

15. A synthetic peptide comprising at least one epitope of P.195.

16. A method for synthesising at least a portion of P.195 or a peptide comprising at least one epitope thereof, said P.195 or peptide being optionally covalently linked to further peptide sequence, which method comprises the steps of:

a) creating a cDNA or genomic DNA library from Plasmodium falciparum;
b) selecting a probe for P.195 and rendering the said probe radio-active;
c) selecting at least one member of the said library by use of the said probe; and
d) using DNA thus selected from the said library to transform a suitable host which may be used to express the said P.195 or a peptide comprising at least one epitope thereof.

17. A vaccine for inducing immunity to malaria comprising a peptide according to claim 15 or P.195 or a peptide comprising at least one epitope thereof when derived from a DNA sequence according to any of claims 1 to 5 in association with a pharmaceutically acceptable carrier therefor.

18. A vaccine according to claim 17 together with an adjuvant.

19. A vaccine according to either of claims 17 and 18 together with at least one other antimalarial vaccine.

Claims

1. A method for synthesising at least a portion of P.195 or a peptide comprising at least one epitope thereof, said P.195 or peptide being optionally covalently linked to further peptide sequence, which method comprises the steps of:

   a) creating a cDNA or genomic DNA library from Plasmodium falciparum;
   b) selecting a probe for P.195 and rendering the said probe radioactive;
   c) selecting at least one member of the said library by use of the said probe; and
   d) using DNA thus selected from the said library to transform a suitable host which may be used to express the said P.195 or a peptide comprising at least one epitope thereof.

2. A method according to claim 1 wherein the DNA sequence selected substantially encodes the P.195 protein of P. falciparum or a peptide comprising at least one epitope thereof.

3. A method according to claim 1 wherein the DNA sequence selected comprises at least a part of the DNA sequence shown in Figure 1 of the accompanying drawings.

4. A method according to claim 1 wherein the DNA sequence possesses substantially the restriction sites as shown in Figures 2 and 3 of the accompanying drawings.

5. A method according to claim 1 wherein the DNA sequence substantially encodes any one of the P.195 fragments occurring in vivo.

6. A method according to claim 1 wherein the DNA sequence selected substantially encodes the naturally occurring 42,000 MW fragment of P.195 or a peptide comprising at least one epitope thereof.

7. A method according to any of claims 1 to 6 wherein the host is transferred by a vector comprising at least the selected DNA sequence.

8. A method according to claim 7 in which the selected DNA sequence is tandemly linked to an amino-terminal coding portion of a gene translatable by the said host and the peptide expressed is in the form of a fusion protein.

9. A method according to claim 8 in which the vector further comprises at least one control sequence.

10. A method according to claim 7 or claim 8 in which the vector further contains at least one control sequence to regulate the expression of the selected DNA sequence in the said host.

11. A method according to claim 7 in which the said vector further comprises a gene capable of translation in the said host into which is inserted the selected DNA sequence suitably altered such that the portion of said gene carboxy-terminal to said DNA sequence is correctly translated on expression in the said host to form a fusion protein comprising at least a part of P.195 or a peptide comprising at least one epitope thereof and at least a part of said gene-encoded protein.

12. A method according to claim 11 in which the vector further comprises at least one control sequence.

13. A method for synthesising a peptide comprising at least one epitope of P.195 having at least part of the sequence shown in figure 1 which method comprises chemical synthesis.

14. A method for inducing immunity to malaria comprising administration of a vaccine containing P.195 or a peptide comprising at least one epitope thereof when produced by a method according to any of claims 1 to 13, in association with a pharmaceutically acceptable carrier therefor.

15. A method according to claim 14 in which the vaccine further comprises an adjuvant.

16. A method according to either of claims 14 and 15 in which the vaccine is administered together with at least one other antimalarial vaccine.

```
TATATATATATACATATGTGTAAGGAAAATAATTTGAATAATATTAAAATTATAGTTATG
        10        20        30        40        50        60

ATGTAATAAATAATTTTTATTATAAAAATAAGGCTAATGTAAAATGCAAAAATAAATGTA
        70        80        90       100       110       120

TACATATTTTTGCTAAGTCATATTTTTAAATTATTAACTTATTTTATTATTATTATTTTT
       130       140       150       160       170       180

                                                M  K  I  I  F  F  L  C  S
ATTTATATATATTATTTATTAGCTTTAATTCAATAATGAAGATCATATTCTTTTTATGTT
       190       200       210       220       230       240

   F  L  F  F  I  I  N  T  Q  C  V  T  H  E  S  Y  Q  E  L  V
CATTTCTTTTTTTTATTATAAATACACAATGTGTAACACATGAAAGTTATCAAGAACTTG
       250       260       270       280       290       300

   K  K  L  E  A  L  E  D  A  V  L  T  G  Y  S  L  F  Q  K  E
TCAAAAAACTAGAAGCTTTAGAAGATGCAGTATTGACAGGTTATAGTTTATTTCAAAAGG
       310       320       330       340       350       360

   K  M  V  L  N  E  G  T  S  G  T  A  V  T  T  S  T  F  G  S
AAAAAATGGTATTAAATGAAGGAACAAGTGGAACAGCTGTTACAACTAGTACACCTGGTT
       370       380       390       400       410       420

   K  G  S  V  A  S  G  G  S  G  G  S  V  A  S  G  G  S  V  A
CAAAGGGTTCAGTTGCTTCAGGTGGTTCAGGTGGCTCAGTTGCTTCAGGTGGCTCAGTTG
       430       440       450       460       470       480

   S  G  G  S  V  A  S  G  G  S  V  A  S  G  G  S  G  N  S  R
CTTCAGGTGGCTCAGTTGCTTCAGGTGGCTCAGTTGCTTCAGGTGGTTCAGGTAATTCAA
       490       500       510       520       530       540

   R  T  N  F  S  D  N  S  S  D  S  D  A  K  S  Y  A  D  L  K
GACGTACAAATCCTTCAGATAATTCAAGTGATTCAGATGCTAAATCTTACGCTGATTTAA
       550       560       570       580       590       600

   H  R  V  R  N  Y  L  L  T  I  K  E  L  K  Y  P  Q  L  F  D
AACACAGAGTACGAAATTACTTGTTAACTATCAAAGAACTCAAATATCCTCAACTCTTTG
       610       620       630       640       650       660

   L  T  N  H  M  L  T  L  C  D  N  I  H  G  F  K  Y  L  I  D
ATTTAACTAATCATATGTTAACTTTGTGTGATAATATTCATGGTTTCAAATATTTAATTG
       670       680       690       700       710       720
```

FIG.1A

```
   G  Y  E  E  I  N  E  L  L  Y  K  L  N  F  Y  F  D  L  L  R
ATGGATATGAAGAAATTAATGAATTATTATATAAATTAAACTTTTATTTTGATTTATTAA
      730       740       750       760       770       780

   A  K  L  N  D  V  C  A  N  D  Y  C  Q  I  F  F  N  L  K  I
GAGCAAAATTAAATGATGTATGTGCTAATGATTATTGTCAAATACCTTTCAATCTTAAAA
      790       800       810       820       830       840

   R  A  N  E  L  D  V  L  K  K  L  V  F  G  Y  R  K  F  L  D
TTCGTGCAAATGAATTAGACGTACTTAAAAAACTTGTGTTCGGATATAGAAAACCATTAG
      850       860       870       880       890       900

   N  I  K  D  N  V  G  K  M  E  D  Y  I  K  K  N  K  K  T  I
ACAATATTAAAGATAATGTAGGAAAAATGGAAGATTACATTAAAAAAAATAAAAAAACCA
      910       920       930       940       950       960

   E  N  I  N  E  L  I  E  E  S  K  K  T  I  D  K  N  K  N  A
TAGAAAATATAAATGAATTAATTGAAGAAAGTAAGAAAACAATTGATAAAAATAAGAATG
      970       980       990      1000      1010      1020

   T  K  E  E  E  K  K  K  L  Y  Q  A  Q  Y  D  L  S  I  Y  N
CAACTAAAGAAGAAGAAAAAAAAAAATTATACCAAGCTCAATATGATCTTTCTATTTACA
     1030      1040      1050      1060      1070      1080

   K  Q  L  E  E  A  H  N  L  I  S  V  L  E  K  R  I  D  T  L
ATAAACAATTAGAAGAAGCACATAATTTAATAAGCGTTTTAGAAAAACGTATTGACACTT
     1090      1100      1110      1120      1130      1140

   K  K  N  E  N  I  K  E  L  L  D  K  I  N  E  I  K  N  F  F
TAAAAAAAAATGAAAACATTAAGGAATTACTTGATAAGATAAATGAAATTAAAAATCCCC
     1150      1160      1170      1180      1190      1200

   P  A  N  S  G  N  T  P  N  T  L  L  D  K  N  K  K  I  E  E
CACCGGCCAATTCTGGAAATACACCAAATACTCTCCTTGATAAGAACAAAAAAATCGAGG
     1210      1220      1230      1240      1250      1260

   H  E  K  E  I  K  E  I  A  K  T  I  K  F  N  I  D  S  L  F
AACACGAAAAAGAAATAAAAGAAATTGCCAAAACTATTAAATTTAATATTGATAGTTTAT
     1270      1280      1290      1300      1310      1320

   T  D  P  L  E  L  E  Y  Y  L  R  E  K  N  K  N  I  D  I  S
TTACTGATCCACTTGAATTAGAATACTATTTAAGAGAAAAAAATAAAAATATTGATATAA
     1330      1340      1350      1360      1370      1380
```

FIG.1B

```
   A  K  V  E  T  K  E  S  T  E  P  N  E  Y  P  N  G  V  T  Y
GTGCAAAGGTTGAAACAAAGGAATCAACTGAACCCAATGAATATCCAAATGGAGTTACTT
      1390       1400       1410       1420       1430       1440

   P  L  S  Y  N  D  I  N  N  A  L  N  E  L  N  S  F  G  D  L
ATCCTTTGTCATATAACGATATTAACAATGCTTTAAATGAACTTAATTCTTTTGGTGATT
      1450       1460       1470       1480       1490       1500

   I  N  P  F  D  Y  T  K  E  P  S  K  N  I  Y  T  D  N  E  R
TAATTAATCCATTTGATTATACAAAAGAACCAAGTAAAAACATATATACTGATAATGAAA
      1510       1520       1530       1540       1550       1560

   K  K  F  I  N  E  I  K  E  K  I  K  I  E  K  K  K  I  E  S
GAAAAAAATTCATAAATGAAATTAAGGAAAAAATTAAAATAGAAAAAAAAAAAATTGAAT
      1570       1580       1590       1600       1610       1620

   D  K  K  S  Y  E  D  R  S  K  S  L  N  D  I  T  K  E  Y  E
CTGATAAAAAATCTTACGAAGACAGATCTAAGTCTTTAAATGATATAACAAAAGAATATG
      1630       1640       1650       1660       1670       1680

   K  L  L  N  E  I  Y  D  S  K  F  N  N  N  I  D  L  T  N  F
AAAAATTACTTAATGAAATTTATGATAGCAAATTCAATAATAATATAGATTTAACTAATT
      1690       1700       1710       1720       1730       1740

   E  K  M  M  G  K  R  Y  S  Y  K  V  E  K  L  T  H  P  N  T
TCGAAAAAATGATGGGTAAAAGATATTCATATAAAGTTGAGAAACTTACACACCCTAATA
      1750       1760       1770       1780       1790       1800

   F  A  S  Y  E  N  S  K  H  N  L  E  K  L  T  K  A  L  K  Y
CTTTTGCATCCTATGAAAATTCTAAACATAATCTTGAAAAGTTAACAAAAGCTCTTAAAT
      1810       1820       1830       1840       1850       1860

   M  E  D  Y  S  L  R  N  I  V  V  E  K  E  L  K  Y  Y  K  N
ATATGGAAGATTATTCTTTAAGGAATATAGTAGTTGAAAAAGAATTAAAATATTATAAAA
      1870       1880       1890       1900       1910       1920

   L  I  S  K  I  E  N  E  I  E  T  L  V  E  N  I  K  K  D  E
ATTTAATAAGCAAAATAGAAAATGAGATTGAAACATTAGTTGAAAATATTAAAAAAGATG
      1930       1940       1950       1960       1970       1980

   E  Q  L  F  E  K  K  I  T  K  D  E  N  K  P  D  E  K  I  L
AAGAACAGCTTTTTGAAAAAAAAATTACTAAAGACGAAAATAAACCAGATGAAAAAATTT
      1990       2000       2010       2020       2030       2040
```

FIG.1C

```
   E  V  S  D  I  V  K  V  Q  V  Q  K  V  L  L  M  N  K  I  D
TAGAAGTATCTGACATTGTAAAAGTACAAGTTCAAAAAGTTTTATTAATGAACAAAATTG
      2050       2060       2070       2080       2090       2100

   E  L  K  K  T  Q  L  I  L  K  N  V  E  L  K  H  N  I  H  V
ACGAATTAAAAAAGACTCAATTGATTTTAAAAAATGTAGAATTAAAACATAATATACATG
      2110       2120       2130       2140       2150       2160

   P  N  S  Y  K  Q  E  N  K  Q  E  P  Y  Y  L  I  V  L  K  K
TTCCCAATTCTTACAAACAAGAAAATAAGCAAGAACCTTATTATTTAATTGTGTTGAAAA
      2170       2180       2190       2200       2210       2220

   E  I  D  K  L  K  V  F  M  P  K  V  E  S  L  I  N  E  E  K
AAGAAATTGATAAATTAAAAGTGTTCATGCCTAAGGTAGAATCATTGATAAATGAAGAAA
      2230       2240       2250       2260       2270       2280

   K  N  I  K  T  Q  G  Q  S  D  N  S  E  P  S  T  E  G  E  I
AAAAAAACATAAAAACACAAGGTCAATCGGATAATTCGGAACCATCAACCGAAGGAGAAA
      2290       2300       2310       2320       2330       2340

   T  G  Q  A  T  T  K  P  G  Q  Q  A  G  S  A  L  E  G  D  S
TAACAGGACAAGCAACTACAAAACCTGGACAACAAGCAGGATCTGCTTTAGAAGGAGATT
      2350       2360       2370       2380       2390       2400

   V  Q  A  Q  A  Q  E  Q  K  Q  A  Q  P  P  V  P  V  P  V  P
CAGTACAAGCACAAGCACAAGAACAAAAACAAGCACAACCACCAGTACCAGTACCAGTAC
      2410       2420       2430       2440       2450       2460

   E  A  K  A  Q  V  P  T  P  P  A  P  V  N  N  K  T  E  N  V
CAGAAGCAAAAGCACAAGTCCCAACACCACCAGCACCAGTAAATAATAAAACTGAAAATG
      2470       2480       2490       2500       2510       2520

   S  K  L  D  Y  L  E  K  L  Y  Q  F  L  N  T  S  Y  I  C  H
TTTCCAAATTAGATTATCTTGAAAAATTATATCAATTTTTAAATACTTCATATATATGTC
      2530       2540       2550       2560       2570       2580

   K  Y  I  L  V  S  H  S  T  M  N  E  K  I  L  K  Q  Y  K  I
ACAAATATATTTTGGTTTCACACTCAACTATGAACGAAAAGATATTAAAACAATATAAAA
      2590       2600       2610       2620       2630       2640

   T  K  E  E  E  S  K  L  S  S  C  D  P  L  D  L  L  F  N  I
TTACAAAGGAGGAAGAAAGCAAATTAAGTTCATGTGATCCATTAGACTTATTGTTTAATA
      2650       2660       2670       2680       2690       2700
```

FIG.1D

```
  Q  N  N  I  P  V  M  Y  S  M  F  D  S  L  N  I  V  Y  H  N
TACAAAATAACATACCTGTAATGTATTCTATGTTTGATAGCTTAAACATAGTTTATCACA
      2710       2720       2730       2740       2750       2760

  Y  L  W  V  Y  E  K  E  I  G  Y  V  F  I  L  L  M  E  I  Y
ACTATTTATGGGTTTATGAAAAAGAAATTGGTTATGTATTTATATTACTTATGGAAATTT
      2770       2780       2790       2800       2810       2820

  E  K  E  M  V  C  N  L  Y  K  L  K  D  N  D  K  I  K  N  L
ATGAAAAAGAAATGGTTTGTAATTTATATAAACTTAAGGATAATGACAAAATTAAAAATT
      2830       2840       2850       2860       2870       2880

  L  E  E  A  K  K  V  S  T  S  V  K  T  L  S  S  S  S  M  Q
TATTAGAGGAAGCGAAAAAAGTATCCACATCTGTAAAAACTCTTTCAAGTTCATCAATGC
      2890       2900       2910       2920       2930       2940

  P  L  S  L  T  P  Q  D  K  P  E  V  S  A  N  D  D  T  S  H
AACCATTATCATTAACACCTCAGGATAAACCCGAAGTAAGTGCAAATGATGATACATCAC
      2950       2960       2970       2980       2990       3000

  S  T  N  L  N  N  S  L  K  L  F  E  N  I  L  S  L  G  K  N
ATTCTACAAATTTGAATAATAGTTTAAAATTATTTGAAAACATATTGAGTCTTGGAAAAA
      3010       3020       3030       3040       3050       3060

  K  N  I  Y  Q  E  L  I  G  Q  K  S  S  E  N  F  Y  E  K  I
ACAAAAATATATACCAAGAATTAATAGGTCAAAAAAGTAGTGAAAACTTTTATGAAAAGA
      3070       3080       3090       3100       3110       3120

  L  K  D  S  D  T  F  Y  N  E  S  F  T  N  F  V  K  S  K  A
TATTAAAAGATAGTGATACATTTTATAATGAATCTTTTACAAATTTTGTAAAATCTAAAG
      3130       3140       3150       3160       3170       3180

  D  D  I  N  S  L  N  D  E  S  K  R  K  K  L  E  E  D  I  N
CTGATGATATTAATTCATTGAATGATGAATCAAAAAGGAAGAAATTAGAAGAAGATATTA
      3190       3200       3210       3220       3230       3240

  K  L  K  K  T  L  Q  L  S  F  D  L  Y  N  K  Y  K  L  K  L
ATAAATTAAAAAAAACTTTACAGTTATCATTTGATTTATATAATAAATATAAATTAAAAT
      3250       3260       3270       3280       3290       3300

  E  R  L  F  D  K  K  K  T  V  G  K  Y  K  M  Q  I  K  K  L
TAGAAAGATTATTTGATAAAAAGAAAACAGTTGGTAAATATAAAATGCAAATTAAAAAAC
      3310       3320       3330       3340       3350       3360
```

FIG.1E

```
   T  L  L  K  E  Q  L  E  S  K  L  N  S  L  N  N  P  K  H  V
TTACTTTATTAAAAGAACAATTAGAATCAAAATTGAATTCACTTAATAACCCAAAGCATG
      3370       3380       3390       3400       3410       3420

   L  Q  N  F  S  V  F  F  N  K  K  K  E  A  E  I  A  E  T  E
TATTACAAAACTTTTCTGTTTTCTTTAACAAAAAAAAAGAAGCTGAAATAGCAGAAACTG
      3430       3440       3450       3460       3470       3480

   N  T  L  E  N  T  K  I  L  L  K  H  Y  K  G  L  V  K  Y  Y
AAAACACATTAGAAAACACAAAAATATTATTGAAACATTATAAAGGACTTGTTAAATATT
      3490       3500       3510       3520       3530       3540

   N  G  E  S  S  P  L  K  T  L  S  E  E  S  I  Q  T  E  D  N
ATAATGGTGAATCATCTCCATTAAAAACTTTAAGTGAAGAATCAATTCAAACAGAAGATA
      3550       3560       3570       3580       3590       3600

   Y  A  S  L  E  N  F  K  V  L  S  K  L  E  G  K  L  K  D  N
ATTATGCCAGTTTAGAAAACTTTAAAGTATTAAGTAAATTAGAAGGAAAATTAAAGGATA
      3610       3620       3630       3640       3650       3660

   L  N  L  E  K  K  K  L  S  Y  L  S  R  G  L  H  H  L  I  A
ATCTAAATTTAGAAAAGAAAAAATTATCATACTTATCAAGAGGTTTACATCATTTAATTG
      3670       3680       3690       3700       3710       3720

   E  L  K  E  V  I  K  N  K  N  Y  T  G  N  S  P  S  V  N  N
CTGAATTAAAAGAAGTAATAAAAAATAAAAATTATACAGGTAATTCTCCAAGCGTAAATA
      3730       3740       3750       3760       3770       3780

   T  D  V  N  N  A  L  E  S  Y  K  K  F  L  P  E  G  T  D  V
ATACGGATGTTAACAATGCATTAGAATCTTACAAAAAATTTCTCCGAGAAGGAAGAGATG
      3790       3800       3810       3820       3830       3840

   A  T  V  V  S  E  S  G  S  D  T  L  E  Q  S  Q  P  K  K  P
TTGCAACAGTTGTAAGTGAAAGTGGATCCGACACATTAGAACAAAGTCAACCAAAGAAAC
      3850       3860       3870       3880       3890       3900

   A  S  T  H  V  G  A  E  S  N  T  I  T  T  S  Q  N  V  D  D
CAGCATCAACTCATGTAGGAGCAGAGTCTAACACAATAACAACATCACAAAATGTCGATG
      3910       3920       3930       3940       3950       3960

   E  V  D  D  V  I  I  V  L  I  F  G  E  S  E  E  D  Y  D  D
ATGAAGTAGATGACGTAATCATAGTACTCATATTTGGAGAATCCGAAGAAGATTATGATG
      3970       3980       3990       4000       4010       4020
```

FIG.1F

```
   L  G  Q  V  V  T  G  E  A  V  T  T  S  V  I  D  N  I  L  S
ATTTAGGACAAGTAGTAACAGGAGAAGCAGTAACTACTTCCGTAATTGATAACATACTTT
      4030       4040       4050       4060       4070       4080

   K  I  E  N  E  Y  E  V  L  Y  L  K  F  L  A  G  V  Y  R  S
CTAAAATTGAAAATGAATATGAGGTTTTATATTTAAAACCTTTAGCAGGTGTTTATAGAA
      4090       4100       4110       4120       4130       4140

   L  K  K  Q  L  E  N  N  V  M  T  F  N  V  N  V  K  D  I  L
GTTTAAAAAAACAATTAGAAAATAACGTTATGACATTTAATGTTAATGTTAAGGATATTT
      4150       4160       4170       4180       4190       4200

   N  S  R  F  N  K  R  E  N  F  K  N  V  L  E  S  D  L  I  F
TAAATTCACGATTTAATAAACGTGAAAATTTCAAAAATGTTTTAGAATCAGATTTAATTC
      4210       4220       4230       4240       4250       4260

   Y  K  D  L  T  S  S  N  Y  V  V  K  D  F  Y  K  F  L  N  K
CATATAAAGATTTAACATCAAGTAATTATGTTGTCAAAGATCCATATAAATTTCTTAATA
      4270       4280       4290       4300       4310       4320

   E  K  R  D  K  F  L  S  S  Y  N  Y  I  K  D  S  I  D  T  D
AAGAAAAAAGAGATAAATTCTTAAGCAGTTATAATTATATTAAGGATTCAATAGATACGG
      4330       4340       4350       4360       4370       4380

   I  N  F  A  N  D  V  L  G  Y  Y  K  I  L  S  E  K  Y  K  S
ATATAAATTTTGCAAATGATGTTCTTGGATATTATAAAATATTATCCGAAAAATATAAAT
      4390       4400       4410       4420       4430       4440

   D  L  D  S  I  K  K  Y  I  N  D  K  Q  G  E  N  E  K  Y  L
CAGATTTAGATTCAATTAAAAAATATATCAACGACAAACAAGGTGAAAATGAGAAATACC
      4450       4460       4470       4480       4490       4500

   P  F  L  N  N  I  E  T  L  Y  K  T  V  N  D  K  I  D  L  F
TTCCCTTTTTAAACAATATTGAGACCTTATATAAAACAGTTAATGATAAAATTGATTTAT
      4510       4520       4530       4540       4550       4560

   V  I  H  L  E  A  K  V  L  N  Y  T  Y  E  K  S  N  V  E  V
TTGTAATTCATTTAGAAGCAAAAGTTCTAAATTATACATATGAGAAATCAAACGTAGAAG
      4570       4580       4590       4600       4610       4620

   K  I  K  E  L  N  Y  L  K  T  I  Q  D  K  L  A  D  F  K  K
TTAAAATAAAAGAACTTAATTACTTAAAAACAATTCAAGACAAATTGGCAGATTTTAAAA
      4630       4640       4650       4660       4670       4680
```

FIG.1G

```
   N  N  N  F  V  G  I  A  D  L  S  T  D  Y  N  H  N  N  L  L
AAAATAACAATTTCGTTGGAATTGCTGATTTATCAACAGATTATAACCATAATAACTTAT
      4690       4700       4710       4720       4730       4740

   T  K  F  L  S  T  G  M  V  F  E  N  L  L  K  S  V  L  S  N
TGACAAAGTTCCTTAGTACAGGTATGGTTTTTGAAAACTTGCTAAAATCCGTTTTATCTA
      4750       4760       4770       4780       4790       4800

   L  L  D  W  K  L  A  R  Y  V  K  H  F  T  T  P  M  R  K  K
ATTTACTTGATTGGAAACTTGCAAGGTATGTTAAACATTTCACAACACCAATGCGTAAAA
      4810       4820       4830       4840       4850       4860

   T  M  I  Q  Q  S  S  G  C  F  R  H  L  D  E  R  E  E  C  K
AAACAATGATCCAACAGAGTTCTGGATGTTTCAGACATTTAGATGAAAGAGAAGAATGTA
      4870       4880       4890       4900       4910       4920

   C  L  L  N  Y  K  Q  E  G  S  K  C  V  E  N  S  N  F  T  C
AATGTTTATTAAATTACAAACAAGAAGGTAGTAAATGTGTTGAAAATTCAAATCCTACTT
      4930       4940       4950       4960       4970       4980

   N  E  N  N  G  G  C  D  A  D  A  K  C  T  E  E  D  S  G  S
GTAACGAAAATAATGGTGGATGTGATGCAGATGCCAAATGTACCGAAGAAGATTCAGGTA
      4990       5000       5010       5020       5030       5040

   N  G  K  K  I  T  C  E  C  T  K  P  D  C  Y  P  L  S  M  V
GCAACGGAAAGAAAATCACATGTGAATGTACTAAACCTGATTGTTATCCACTTTCGATGG
      5050       5060       5070       5080       5090       5100

   I  F  C  S  S  S  N  F  L  G  I  S  F  L  L  I  L  M  L  I
TAATTTTCTGCAGTTCCTCTAACTTCTTAGGAATATCATTCTTATTAATACTCATGTTAA
      5110       5120       5130       5140       5150       5160

   L  Y  S  F  I
TATTATACAGTTTCATTTAAAAAATGTAGGAGTTAAAATATGTTACCTTAATTTTTTTTT
      5170       5180       5190       5200       5210       5220

TTTTTTTTTTTTTTAAATATATATATATATTAATATATATATATAAAATATTACATAATA
      5230       5240       5250       5260       5270       5280

TATATATATATATTTAGTTATTACAGGAATAGTGATATTTTAGTCATGTTCAAAATATAT
      5290       5300       5310       5320       5330       5340
```

FIG.1H

```
TAAAAAATTATAAATATTATAATAAAAAAAAAAAAAAAAAAAATTATATACTTATAAAT
      5350      5360      5370      5380      5390      5400

TTATACATTTATACATATATATATATATATTTTTTTTTTCTTCTTTCTTTTCAAGTTCTAT
      5410      5420      5430      5440      5450      5460

TTTATATTTTATATATAGATTTAATAAAAAACTTTTTAAAATAAAAAAAAGTACGTAAAT
      5470      5480      5490      5500      5510      5520

TTTAATATATATATATATATATATAATATATATATAATATATATTTATTTATTTATATGT
      5530      5540      5550      5560      5570      5580

ATAATATATTTACATATATTATTATAATATATATATTTTTTTTTTACGCATACATAAAAAG
      5590      5600      5610      5620      5630      5640

CATTTTTTTTTTTTATAAACATTCCAACAATTATAAAATAACTTTAATAATAACATTAAA
      5650      5660      5670      5680      5690      5700

TTTTTATTTTTTTTTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAACTAAAGAGATTATTCA
      5710      5720      5730      5740      5750      5760
```

FIG.1I

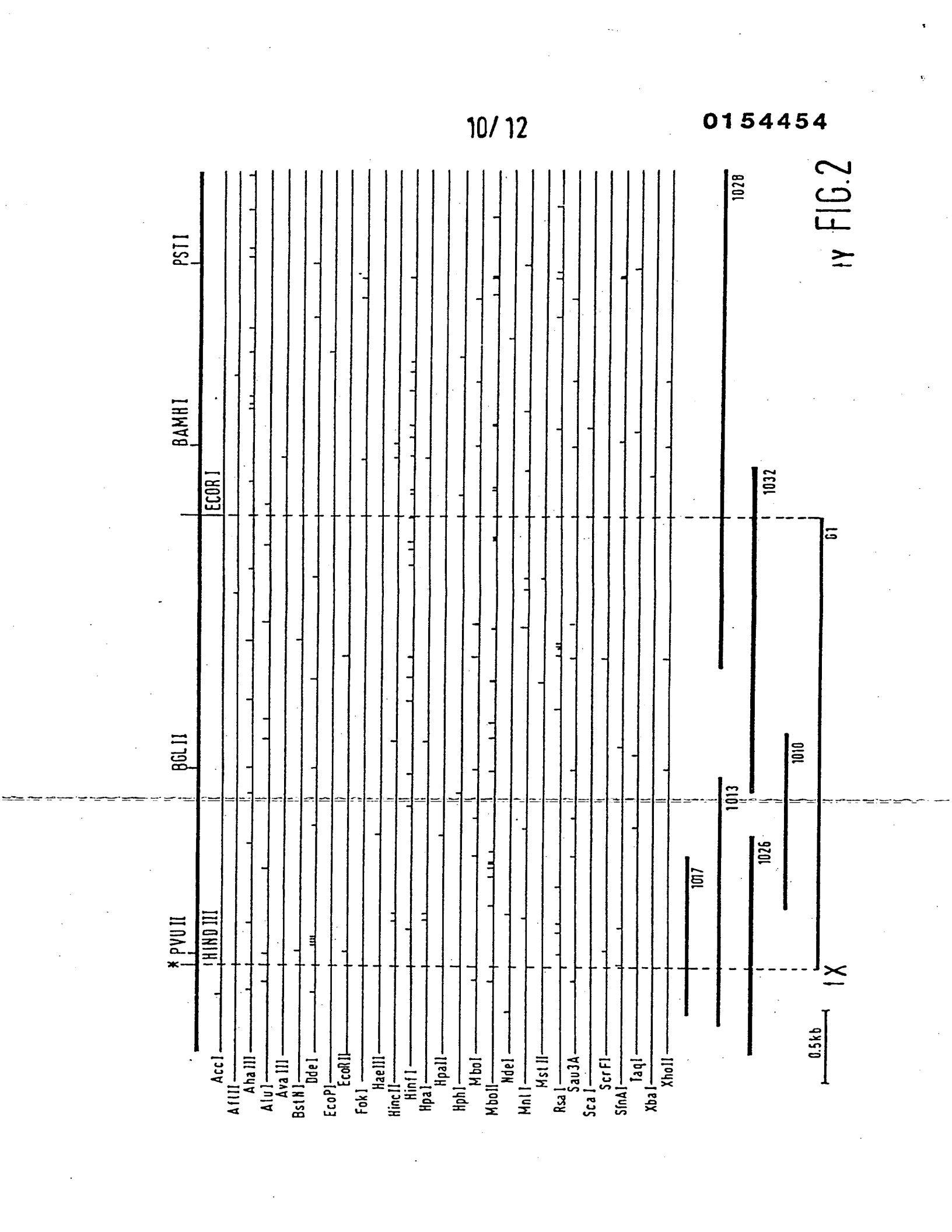

* PVU II
HIND III
BGL II
BAMH I
ECOR I
PST I
Acc I
Afl III
Aha III
Alu I
Ava III
BstN I
Dde I
EcoP I
EcoR II
Fok I
Hae III
Hinc II
Hinf I
Hpa I
Hpa II
Hph I
Mbo I
Mbo II
Nde I
Mnl I
Mst II
Rsa I
Sau 3A
Sca I
Scr FI
Sfn A I
Taq I
Xba I
Xho II
1017
1013
1026
1010
1032
1028
61
1X
1Y
0.5kb

FIG.2

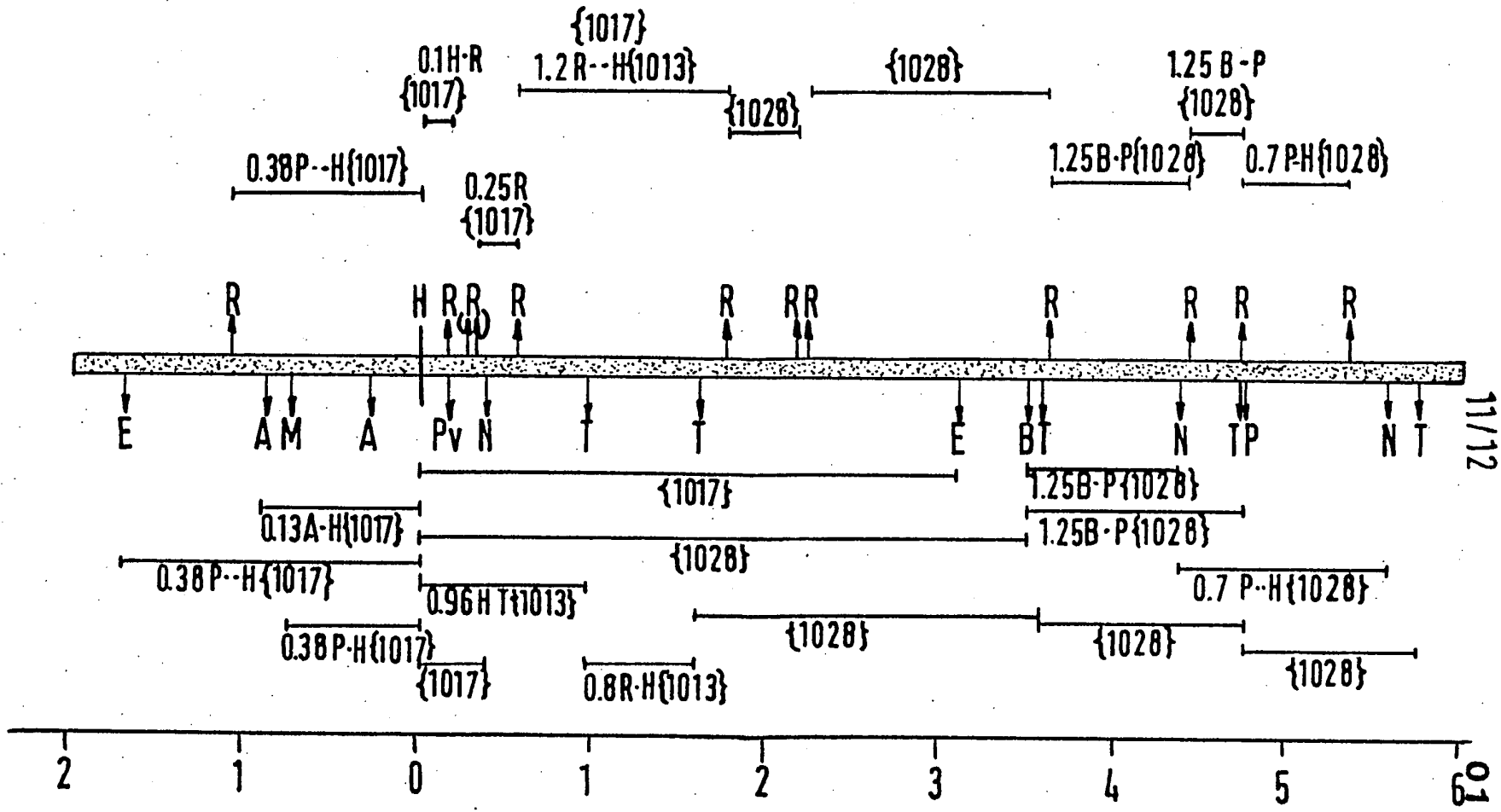


FIG.3

11/12

0154454

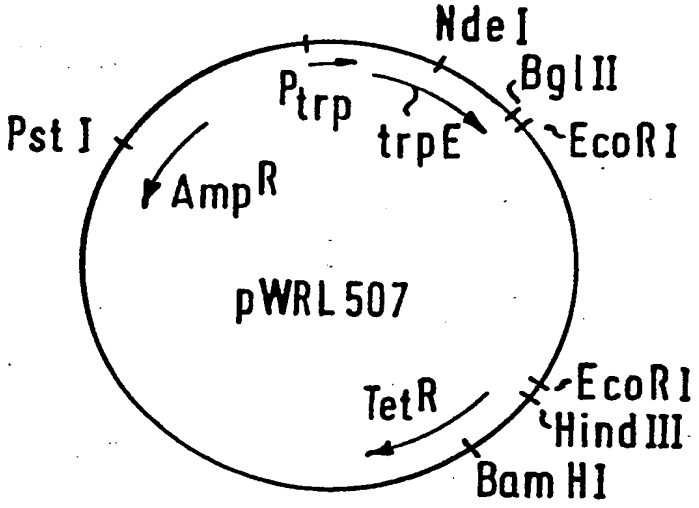
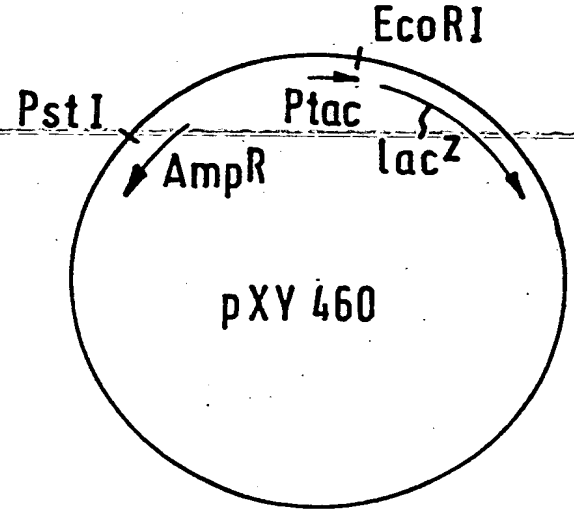

Nde I
Bgl II
Ptrp
trpE
EcoR I
Pst I
AmpR
pWRL 507
EcoR I
TetR
Hind III
Bam HI

FIG. 4


EcoRI
Pst I
Ptac
AmpR
lacZ
pXY 460

European Patent Office

EUROPEAN SEARCH REPORT

0154454

Application number

EP 85301173.2

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int Cl 4)
P,A	WO - A1 - 84/02 917 (THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH) * Claims 1-3,8,10,24,25 * --	1,7,9-12,17,18	C 12 N 15/00 C 12 P 19/34 C 12 P 21/00 C 07 K 15/04 A 61 K 39/015 C 07 K 17/00 //C 12 R 1:90
D,P	NATURE, vol. 311, no. 5984, September 27, 1984 (London, New York) R. HALL et al. "Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria" pages 379-382 * Totality * --	1	
A	EP - A2 - 0 062 924 (THE WELLOME FOUNDATION LIMITED) * Claims 1,4,8 * ----	1,17,18	

TECHNICAL FIELDS SEARCHED (Int Cl 4)

C 12 N
C 12 P
C 07 K
A 61 K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	21-05-1985	WOLF

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO Form 1503 03.82